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Pharmacological inhibitors of NAD(P)H quinone oxidoreductase, NQO1: Structure/activity relationships and functional activity in tumour cells

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ABSTRACT

NAD(P)H quinone oxidoreductase (NQO1) has multiple functions in the cell including an ability to act as a detoxifying enzyme and as a protein chaperone. The latter property is particularly important in oncology as one of the client proteins of NQO1 is p53. The inhibitor, dicoumarol, is classically used to probe the biological properties of NQO1, but interpretation of enzyme function is compromised by the multiple "off-target" effects of this agent. Coumarin-based compounds that are more potent than dicoumarol as inhibitors of recombinant human NQO1 have been identified (Nolan et al., J Med Chem 2009;52:7142–56) The purpose of the work reported here is to demonstrate the functional activity of these agents for inhibiting NQO1 in cells. To do this, advantage was taken of the NQO1-mediated toxicity of the chemotherapeutic drug EO9 (Apaziquone). The toxicity of this drug is substantially reduced when the function of NQO1 is inhibited and many of the coumarin-based compounds are more efficient than dicoumarol for inhibiting EO9 toxicity. The ability to do this appears to be related to their capacity to inhibit NQO1 in cell free systems. In conclusion, agents have been identified that may be more pharmacologically useful than dicoumarol for probing the function of NQO1 in cells and tissues.

1. Introduction

NAD(P)H quinone oxidoreductase (NQO1, DT-diaphorase, quinone reductase 1, vitamin K reductase, E.C.1.6.99.2) is a ubiquitous, homodimeric flavoprotein with one molecule of FAD non-covalently bound per monomer [1]. NQO1 is generally regarded as a detoxifying enzyme, where its major role has been considered to be its ability to catalyse the reduction of various quinones [2]. NQO1 has also been shown to act as a chaperone, thereby stabilising various proteins, including the tumour suppressor protein p53 [3] and other short-lived proteins such as ornithine decarboxylase [4]. This stabilisation phenomenon is NAD(P)H dependent [5,6], which suggests that the binding of NQO1 to its client protein(s) is most efficient when the enzyme-bound FAD is in its reduced form. Dicoumarol is the most commonly used inhibitor of NQO1, and it acts through competitive binding with NAD(P)H and thereby prevents the two-electron transfer to FAD from occurring. Hence, addition of dicoumarol to cells has been shown to result in the targeting of p53 for degradation [7].

The pharmacological action of dicoumarol for interfering with the function of NQO1 is compromised by extensive protein binding [8] and confounded by "off-target" effects such as the increased production of intracellular superoxide [9] and mitochondrial uncoupling [10]. Thus, we have sought to develop novel inhibitors of NQO1 which retain the inhibitory potency of dicoumarol but lack its unfavourable off-target effects. To achieve this the National Cancer Institute compound database was mined to identify "dicoumarol-like" molecules that could act as competitive inhibitors of NQO1 [11]. From these studies, a computational and synthetic platform was generated to identify novel coumarin-based competitive NQO1 inhibitors, with the aim of discovering agents with potentially superior pharmacological properties to dicoumarol [12].

Previously, NQO1 has been used as the target enzyme in tumour cells to exemplify the "enzyme directed" approach to anticancer drug development [13]. Arguably, the best example of this was in the development of EO9 (5-aziridinyl-3-hydroxymethyl-2-(3-hydroxyprop-1-enyl)-1-methylindole-4,7-dione, Apaziquone). Early work clearly demonstrated a relationship between the toxicity of EO9 and intracellular activity of NQO1 [14–16]. The function of NQO1 was to metabolically activate EO9 to generate

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highly cytotoxic species [17,18] and this process was inhibited by dicoumarol [19,20]. Thus, in order to establish the potency and functional ability of the novel coumarin-based compounds as inhibitors of NQO1 in cells, their ability to inhibit EO9 toxicity was evaluated. The results provide a cell-based structure/activity relationship relating enzyme inhibitory potency in cell free systems with the ability to inhibit EO9 toxicity; and further, compounds are identified that are more active than dicoumarol as inhibitors of NQO1 in cells.

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 described by Nolan et al. [12]. They comprise two series of substituted coumarins; the first includes a set of substituted dicoumarol analogues (the symmetric, **S** series), 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 up to 100 mM. The cytotoxin EO9, was synthesized in-house, using previously described methods [21].

2.2. Cell lines, cell culture and NQO1 activity

HT29 colon carcinoma, A549 non-small cell lung cancer and MIA PaCa-2 pancreatic carcinoma cells were chosen for use in the current work as they have previously been reported to have high levels of functional NQO1 [14,16]. HT29 and A549 cells 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); MIA PaCa-2 cells were grown in Dulbecco's Modified Eagle Medium (Invitrogen) supplemented with 10% (v/v) FCS and 2.5% (v/v) horse serum (Invitrogen) plus 2 mM L-glutamine. The cells were maintained at 37 °C in a humidified incubator in an atmosphere of air plus 5% CO_2. NQO1 activity in the three cell lines was measured as previously described [11,22]. Briefly, cell lysates were prepared and added to reaction mixtures, at pH 7.5, containing 200 μM NADH, 70 μM cytochrome *c* and 20 μM menadione as the terminal electron acceptor. Reactions were carried out at 25 °C and cytochrome *c* reduction was followed at 550 nm. The activity of NQO1 in the cell lines was defined as the difference in rates of reduction of cytochrome *c*/min/mg lysate protein obtained in the presence and absence of 100 μM dicoumarol.

2.3. Impact of NQO1 inhibition on EO9 toxicity

HT29, A549 and MIA PaCa-2 cells were seeded into 96-well plates, each at 7500 cells/ml, and left to adhere overnight. The cells were then given dicoumarol or the other compounds from the **AS** or **S** series (all at 200 μM) together with varying concentrations of EO9 from 0.1–10,000 nM for 3 h. For none of the potential inhibitors, including dicoumarol, was a concentration of 200 μM toxic over a 3 h period. After this time, the media was removed, cells washed once in PBS (OXOID, Hampshire, UK) and fresh media added. The plates were incubated for a further 96 h and toxicity assessed using the MTT assay [23]. Briefly, 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

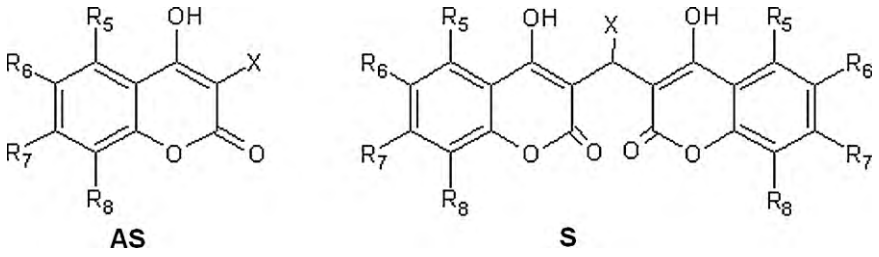

Fig. 1. Structures of the substituted 4-hydroxycoumarins (**AS** series) and analogues of dicoumarol (**S** series). Substituents R5–R8 and X are given in Table 1.

Table 1
Substituents in the **S** and **AS** series (Fig. 1) together with the concentration of each compound that inhibits the activity of purified human NQO1 by 50% (IC_{50}). Values of IC_{50} were obtained by carrying out the enzyme activity assay in the presence or absence of 0.14% (w/v) BSA [12]. Also given are values (±SD) of the concentration of each inhibitor to cause 50% toxicity in HT29 cells in the presence of 1 μM EO9. This concentration of EO9 alone will kill 90% of the cells; hence the values quoted give an estimate of the efficiency of protection against EO9 toxicity. This reflects the ability of the **S** and **AS** compounds to inhibit NQO1 activity in HT29 cells.

ID	R5	R6	R7	R8	X	IC_{50} (nM) No BSA	IC_{50} (nM) With BSA	Concentration required to protect against EO9 toxicity (μM)
S1	H	H	H	H	H	2.6 ± 1.6	404 ± 184	211 ± 83
S3	OCH_3	H	H	H	H	2.8 ± 0.42	38 ± 2.1	18 ± 9.0
S4	H	OCH_3	H	H	H	11 ± 3.9	3300 ± 600	>1000
S5	H	H	OCH_3	H	H	6.0 ± 3.2	790 ± 355	875 ± 211
S10	H	CH_3	CH_3	H	H	0.41 ± 0.38	233 ± 68	106 ± 29
S11	H	OCH_3	OCH_3	H	H	62 ± 32	1497 ± 442	1954 ± 940
S13	H	H	CH_3	CH_3	H	0.42 ± 0.14	149 ± 101	132 ± 9.8
AS1	H	CH_3	CH_3	H	1-Naphthyl	7.7 ± 4.5	1095 ± 290	105 ± 77
AS2	H	CH_3	CH_3	H	2-Naphthyl	2.5 ± 1.9	167 ± 83	50 ± 33
AS3	H	CH_3	CH_3	H	Phenyl	39 ± 12	660 ± 108	128 ± 69
AS4	H	H	7,8-C_4H_4		1-Naphthyl	6.3 ± 2.7	450 ± 325	58 ± 26
AS5	H	H	7,8-C_4H_4		2-Naphthyl	2.2 ± 1.6	225 ± 151	97 ± 52
AS6	H	H	7,8-C_4H_4		Phenyl	35 ± 21	880 ± 364	133 ± 41
AS14	H	CH_3	CH_3	H	3,4 Dimethylbenzyl	9.9 ± 4.4	192 ± 41	62 ± 19

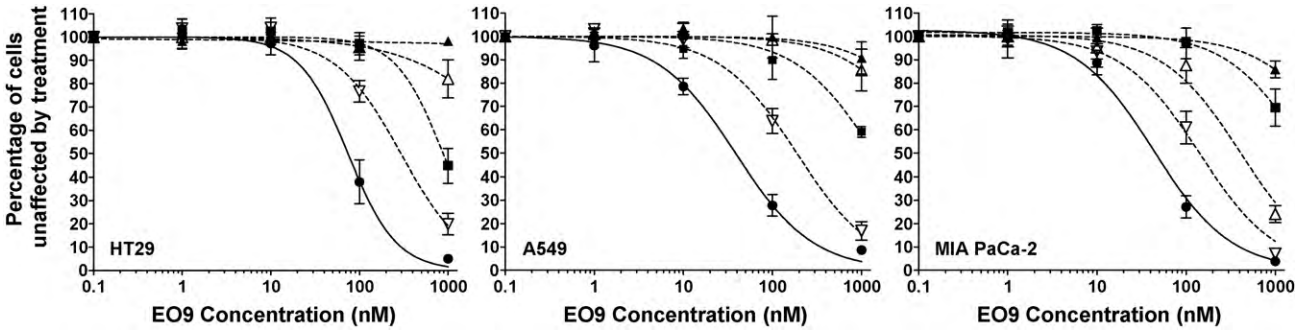

Fig. 2. HT29, A549 and MIA PaCa-2 cells were treated for 3 h with varying concentrations of EO9 and 200 μM of some of the novel **S** and **AS** compounds. Surviving cells were then allowed to grow for 96 h and toxicity then assessed using the MTT assay. Each point represents the mean of at least three independent experiments. EO9 alone = (●), EO9 + **S1** (dicoumarol) = (■), EO9 + **S3** = (▲), EO9 + **S4** = (▽), EO9 + **AS1** = (△).

of 1.5 mM. Cells were incubated with MTT for 4 h, media was removed and the remaining formazan crystals dissolved in DMSO. Absorbance at 540 nm was then measured using a multi-well scanning spectrophotometer. Values of IC_{50} were calculated as the concentration of EO9 required to reduce optical density by 50% relative to vehicle treated control cells. Since optical density is directly proportional to cell density [23], these values of IC_{50} can be regarded as the concentration of compound(s) required to reduce proliferating by 50%. All toxicity experiments were repeated on at least three separate occasions. Data were analyzed and curves drawn using the GraphPad Prism 5 software package.

To determine the potency at which dicoumarol or the other compounds in the **AS** or **S** series are able to inhibit the NQO1-mediated toxicity of EO9, cells were treated for 3 h with varying concentrations of the putative inhibitors together with a fixed dose of 1 μM EO9. As above, the cells were then washed with PBS and fresh media was added. The plates were incubated for 96 h and the MTT assay was used to determine cell viability. A concentration of 1 μM EO9 will kill >90% of HT29, A549 and MIA PaCa-2 cells when exposed for 3 h, hence protection against toxicity will indicate inhibition of intracellular NQO1 activity. The potency of the different inhibitors was defined as the concentration of agent, which in the presence of 1 μM EO9, caused only 50% cell kill.

3. Results

The dicoumarol analogues (the **AS** and **S** series) have previously been identified as potent inhibitors of NQO1 in cell free assays, where inhibitory potency correlated with a computationally derived binding affinity [12]. It was also apparent that the compounds can be extensively protein bound as was evidenced by comparison of values of IC_{50} for enzyme inhibition obtained in the absence or presence of 0.14% w/v BSA; with changes in IC_{50} varying from 13.5-fold to in excess of 10,000 (Table 1 and [12]). Since protein binding is obviously an important factor for the potential pharmacological activity of these putative inhibitors, a subset of compounds have been taken from the series and evaluated for their ability to inhibit the toxicity of EO9 in three different cell lines. The ability to modulate EO9 toxicity can be regarded as a surrogate measure of the pharmacological efficiency of these compounds to act as inhibitors of NQO1 in cells.

The three cell lines have NQO1 activities (±SD) of 1691 ± 715, 3011 ± 532 and 1252 ± 142 nmol cytochrome *c* reduced/min/mg protein for HT29, A549 and MIA PaCa-2 cells respectively. The cells were first treated for 3 h with various concentrations of EO9 with or without a 200 μM concentration of dicoumarol or the **S** and **AS** compounds, then washed and the media was replaced for another 96 h, at which time MTT was used to assess cellular viability. Each of the cell lines are sensitive to treatment with EO9 alone. IC_{50} values (±SD) are 78 ± 38, 33 ± 7.4 and 56 ± 18 nM for the HT29, A549 and MIA PaCa-2 cells respectively, which is consistent with the relatively high expression of NQO1 in each of the cell lines. Dose response curves for cells exposed to EO9 with or without each of the inhibitors (at 200 μM) were established and examples for each cell line are given in Fig. 2. None of the inhibitors showed any toxicity alone at 200 μM and at this concentration all of the NQO1 inhibitors protected against EO9 toxicity. However, as is apparent in Fig. 2 their efficiency for doing this differs markedly. For example, in each cell line, **S3** completely prevents EO9 toxicity, whereas dicoumarol (**S1**), **S4**, and **AS1** are less effective.

In order to quantify the ability of each of the NQO1 inhibitors to be functional in cells, HT29 cells were then treated with 1 μM EO9 and varying concentrations of each of the **AS** and **S** series of compounds. The maximal concentration of each compound tested depended on solubility and/or final concentration of vehicle (usually DMSO) or the toxicity of the inhibitor. 1 μM EO9 alone results in >90% reduction in cell proliferation in HT29 cells; therefore adding increasing concentrations of enzyme inhibitor should elicit a steady decrease in toxicity of EO9. The concentration of the **AS** and **S** compounds needed to inhibit the toxicity of 1 μM EO9 by 50% can then be used as a measure of the ability of the compounds to inhibit NQO1 in cells. Fig. 3 shows representative dose response curves for HT29 cells exposed to EO9 and some of the NQO1 inhibitors, and it is clear that protection efficiency varies between the different compounds. Values of IC_{50} for protection against EO9 toxicity are given in Table 1 and these values differ by over 30-fold. **S3** appears to be the most potent compound for protecting against EO9 toxicity in HT29 cells and in Fig. 4 it is compared with dicoumarol for its ability to protect against the

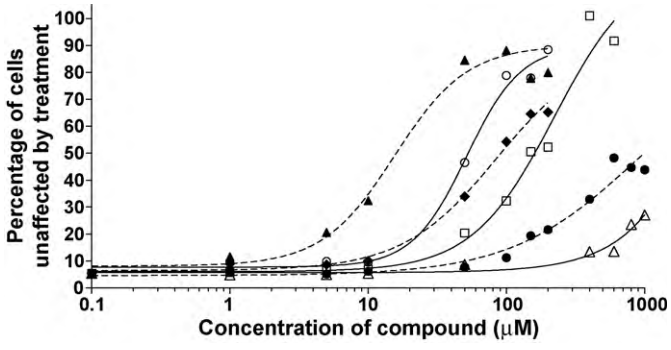

Fig. 3. HT29 cells were treated for 3 h with 1 μM EO9 and varying concentrations of the **S** and **AS** compounds; toxicity was determined 96 h later by the MTT assay. Each point represents the mean of at least three independent experiments. **S1** (dicoumarol) = (□,) **AS1** = (◆), **AS4** = (○), **S3** = (▲), **S4** = (△), **S5** = (●). The concentration of inhibitor to cause 50% toxicity in the presence of 1 μM EO9 is used as the measure of inhibitory potency and these values are recorded in Table 1.

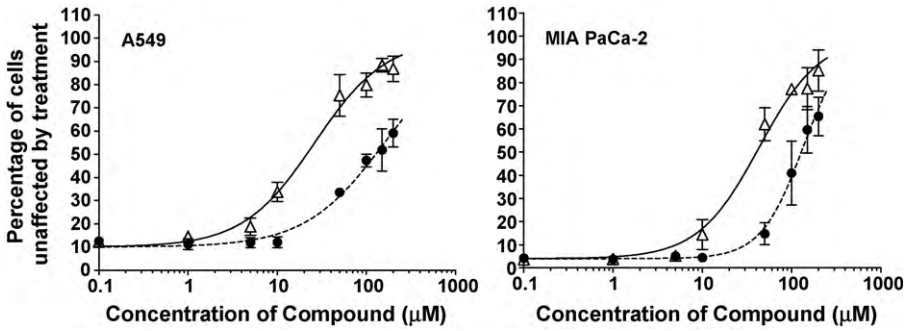

Fig. 4. Comparison of the efficiency of **S1** (dicoumarol), (●), and **S3**, (△), for protecting against the toxicity of 1 μM EO9 in A549 and MIA PaCa-2 cells.

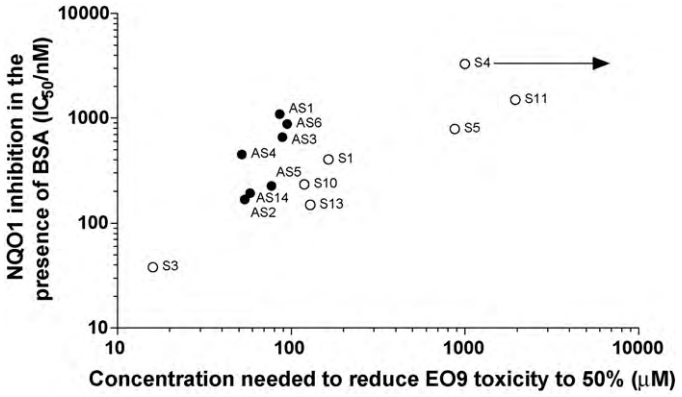

Fig. 5. Relationship between the concentration of the **S** and **AS** compounds required to inhibit recombinant human NQO1 activity by 50% in the presence of 0.14% (w/v) BSA and the concentration of the compounds required to reduce the toxicity of 1 μM EO9 to 50% in HT29 cells. The arrow given for **S4** indicates that the highest concentration tested for this compound was 1 mM (limited by solubility) and that this concentration was insufficient to inhibit toxicity to the 50% level (see Fig. 3) (**AS** compounds = (●); **S** compounds = (○)).

toxic effects of EO9 in A549 and MIA PaCa-2 cells. In each cell line, **S3** is a more efficient compound, with differences in efficiency ranging from 3- in MIA PaCa-2 cells to >10-fold in HT29 cells. Interestingly, **S3** shows equal potency to dicoumarol as an inhibitor of recombinant NQO1 (Table 1) but in the presence of 0.14% (w/v) BSA it is significantly more efficient, presumably because of reduced protein binding. Therefore, the ability of the **AS** and **S** series of compounds to protect against 1 μM EO9 toxicity in HT29 cells was compared with potency for inhibition of the activity of recombinant NQO1 in the presence of BSA. These results are given in Fig. 5 and show a clear trend between enzyme inhibition in a cell free system and inhibition of NQO1-mediated toxicity of EO9 in cells, with **S3** being the most effective inhibitor.

4. Discussion

The pharmacological role of NQO1 has traditionally been assessed using the competitive inhibitor dicoumarol. However, it is extensively protein bound [8,24], which together with a myriad of other "off-target" effects [9,10,25] make its use for evaluating the function of NQO1 in cells and tissues far from optimal. Flavone-8-acetic acid and 5,6-dimethylxanthenone-4-acetic acid have also been shown to competitively inhibit NQO1 [26] but these too show substantial lack of specificity. An alternative approach has been to develop irreversible inhibitors, exemplified by ES936 [27,28]. This agent deactivates NQO1 by alkylating the active site. However, this attractive mechanism-based approach also has limitations regarding target selectivity [29,30]. These observations prompted us to return to the coumarin-based pharmacophore in order to attempt to develop compounds with both greater potency and greater selectivity than dicoumarol for inhibiting NQO1. Using a combined computational and synthetic chemistry approach linked to biochemistry structure was linked with function and in particular, computationally derived binding affinity was correlated with inhibitory potency [12]. This work also confirmed that protein binding could compromise the activity of these agents. However it is clear (as is illustrated in Table 1 when comparing IC_{50} values for recombinant enzyme inhibition in the presence and absence of BSA) that the protein binding varies considerably between the compounds. Hence, in the development of agents which may be pharmacologically useful, the next step was to determine their potency for NQO1 inhibition in cells and this was the primary aim of the work reported here. To achieve this, advantage was taken of the fact that tumour cells expressing high levels of functional NQO1 are exquisitely sensitive to the indolequinone, EO9 [14–16]. This agent, under aerobic conditions, is dependent on NQO1 for metabolism to give active species that can cause toxicity. Interestingly, it is this process that was exploited to develop the mechanism-based inhibitor ES936 [28]. Here it is shown that dicoumarol and each of the 13 coumarin-based analogues can inhibit the toxicity of EO9 in three different cancer cell types, each expressing significant levels of NQO1. A 3 h exposure to 1 μM EO9 was used as a cytotoxic dose and from the data, the concentration of the compounds needed to reduce toxicity to 50% was derived. This was then used as the measure of NQO1 inhibitory potency in cells, and values obtained in HT29 cells are given in Table 1. The efficiency of the different compounds for protecting against EO9 toxicity and hence inhibiting NQO1 in cells varies by over 100-fold, with many of the compounds showing greater efficiency than dicoumarol. Interestingly, inspection of the **S** series (which are dicoumarol analogues) shows a good correlation between the inhibition of NQO1 in cells with enzyme inhibitory potency in a cell free system containing BSA (Fig. 5). For the **AS** series, all the compounds are more efficient than dicoumarol and it would appear their ability to protect against EO9 toxicity is greater than would be predicted from their enzyme inhibition IC_{50} obtained in the presence of BSA.

The biological role of NQO1 can also be evaluated by genetic disruption of the gene. Much of this work has been carried out by Jaiswal and co-workers [6,31–34]. They have generated NQO1 knockout mice and have shown that loss of protein function is associated with increased susceptibility to a variety of carcinogens [31–33] and also that loss of NQO1 is accompanied by a reduction in p53 levels [6,32] and changes in NFκB signalling [34]. These observations have parallels with the findings of Asher et al. [3–5,7] who showed that the dicoumarol-mediated inhibition of NQO1 in cells resulted in the targeting of p53 for degradation. As a consequence, there is likely to be scope for the development of NQO1 inhibitors as potential therapeutics. For example, transient drug-induced deregulation of p53 can result in protection of bone

marrow cells from the cytotoxic effects of radiation [35,36]. Therefore, targeting p53 for degradation via inhibition of NQO1 would be a novel way of achieving this radioprotective effect. In the present work it has been shown that there are a variety of compounds that can inhibit NQO1 more efficiently than dicoumarol and, in addition, there are results to be published elsewhere, showing that many of these compounds lack the "off-target" effects associated with dicoumarol.

In summary, coumarin-based compounds have been identified that have greater efficiency than dicoumarol for inhibiting NQO1 in cells. These agents may well be useful as pharmacological probes of the function of NQO1 in cells and tissues. Furthermore, they may also have therapeutic applications and these are currently under study.

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