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Synthesis of Analogues of Dicoumarol and Their Biological Evaluation

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Abstract

Both symmetrical (**1a-f**) and asymmetrical (**2a-f**) analogues of dicoumarol were synthesized in 20 – 86% yield by using microwave assisted one-pot protocol. Their ability to inhibit NAD (P)H:oxidorectase quinone 1 (NQO1) and cytotoxicity towards A549 small lung cancer cell line were evaluated. Interestingly, (*E*)-3-(2-hydroxynaphthalen-1-y)chroman-2,4-dione (**2d**) showed not only moderate inhibitory potency (IC_{50} = 20 ± 6 nM) towards NQO1 but also was toxic (IC_{50} = 9.2 ± 0.2 μM) towards the A549 small lung cancer cell line.

Keywords: asymmetrical, cytotoxicity, inhibitor, NQO1, symmetrical, synthesis

1. Introduction

NQO1 is a ubiquitous flavoprotein, which functions in a catalytic manner and thus can be called flavoenzyme. NQO1 belongs to a group of enzymes called detoxifying enzymes which protect the cells against toxic metabolites formed during the cell's metabolic processes. It is well known fact that NQO1 functions primarily to protect healthy cells from oxidative stress (Kameyama, et al., 2017) and electrophilic attack (Li, et al., 2015) which could lead to genetic instability and cell apoptosis.

As a detoxifying enzyme, NQO1 catalyses an obligate 2-electron reduction of broad range of substrates such as quinones (Ernester & Navazio, 1958), quinines, imines (De Flora, Bennicelli, D'Agostini, Izzotti, & Camoirano, 1994), nitro and azo compounds (Siegel, et al., 2004). There are evidences in support for a role of NQO1 in the detoxification of quinones (**3a**). This is provided by the observation that dicoumarol (Figure 1), an inhibitor of NQO1 with IC_{50} value = 2.6 nM (Ernester, (1967), enables the toxicity of quinones to hypatocytes (Miller, Rogers, & Cohen, 1986; Thor et al., 1982) and this can only be possible by blocking 2 electron reduction to non-toxic hydroquinone (**3c**) and thus allowing large amount of quinones to be available for one electron reduction to toxic semi-quinone radicals (**3b**) by cytochrome p450 reductases as depicted in Scheme 1.

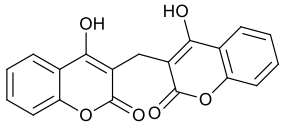
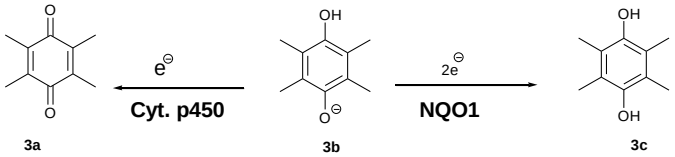


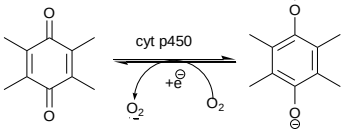
Figure 1. Structure of dicoumarol



Scheme 1. One electron reduction of quinone (**3a**) by cytochrome p450 resulted in the formation of semi-quinone radical (**3b**), while two electron reductions by NQO1 gives hydroquinone (**3c**)

Quinones especially *para*-quinones, belong to a category of ubiquitous and naturally occurring compounds. Quinones obtained from polycyclic aromatic hydrocarbons are found in large quantities in all burnt organic materials such as urban air partivulates, auto-mobile exhaust, cigarette smoke, drugs and many foodstuffs (Driscol, et al., 1974; Chesis, Levin, Smith, Ernester & Ames, 1984). By and large, compounds containing quinoid nucleus are potent as cancer

causing agents. They are highly reactive as electrophiles and also generate unstable semi-quinone radicals *via* one electron reduction by cytochrome p450 reductases. The semi-quinone radicals subsequently undergo redox cycling in the presence of molecular oxygen forming highly reactive oxygen species (ROS) as depicted in Scheme 2.

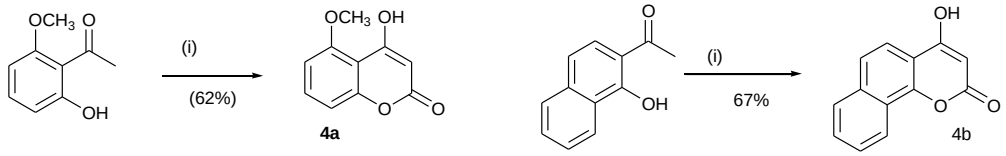


Scheme 2. One electron reduction of quinone to a semi-quinone radical caused by cytochrome p450 reductases and redox cycling in the presence of molecular oxygen forming superoxide (O_2^-) a reactive oxygen species (ROS)

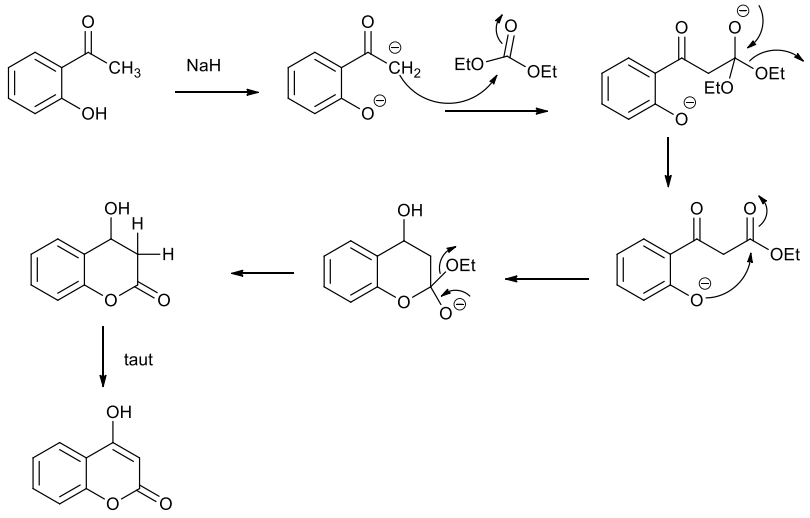
This can lead to oxidative stress and consequently, tissue degeneration, apoptosis, premature aging, cellular transformation and neoplasia according to a research carried out by Schuetzie et al., (1994). Obligate two electron reductions by the NQO1 prevents these harmful effects by producing more stable hydroquinones, which can be removed from the cell by conjugation with glutathione or glucuronic acid and, consequently providing cellular protection (Lind, Hochstein & Ernester, 1982). In view of this, NQO1 has become a potential target in order to enhance the susceptibility of tumor cells to chemotherapeutic drugs. The aim of this research therefore, was to prepare some new inhibitors of NQO1 using the biologically active 4-hydroxycoumarin and its derivatives as precursors (Overmunn et al., 1994; Silvia, 2012).

2. Synthesis

The 4-hydroxycoumarin derivatives (**4a and 4b**) which served as precursors for the target compounds were synthesized by reacting the appropriate acetophenone with diethyl carbonate in the presence of sodium hydride (NaH) as illustrated in Scheme 3. The structural identities of all the cyclised products were confirmed by ^{1}H NMR spectrum which is identical to the data reported by Carberry et al., (1997). The presence of vinylogous carboxylic acid makes these compounds moderately strong acids. The mechanism of this reaction is illustrated in Scheme 4.



Scheme 3. Reaction scheme for the synthesis of derivatives of 4-hydroxycoumarin (**4a and 4b**). Reagents and conditions: (i) diethyl carbonate, sodium hydride, at reflux, 3hr



4c

Scheme 4. Base-mediated cyclisation reaction of 2-hydroxy-6-methoxy acetophenone to give 4-hydroxycoumarin (**4c**)

The condensation of 4-hydroxycoumarin (**4c**) and its derivatives (**4a and 4b**) and the appropriate aromatic aldehydes at a molar ratio of (2:1 and 1:1) as reported in the experimental section using microwave irradiation lead to the formation

of symmetrical dimer (**1a-f**) and asymmetrical (**2a-f**) analogues of dicoumarol respectively as depicted in (Fig 2). The asymmetrical analogues were obtained in poor yields (20-31%) in contrast to symmetrical dimer (65-91%) due to competitive dimer formation.

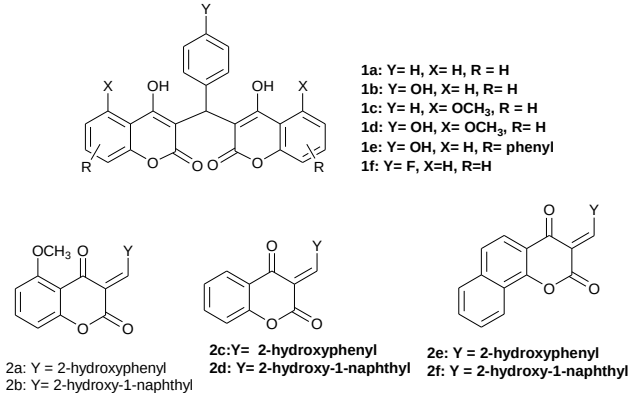


Figure 2. Structures of the target compounds; symmetrical dimer (**1a-f**) and asymmetrical (2**a-f**) analogues of dicoumarol

3. Evaluation of the IC_{50} Values

The IC_{50} value, in terms of an enzyme assay, represents the concentration of a drug that is required for 50% inhibition in *vitro*, whereas, in terms of cytotoxicity, it represents the concentration of a drug required to inhibit the growth of cells by 50%. The symmetrical dimer (**1a-f**) and asymmetrical (**2a-f**) analogues of dicoumarol (Table 1) were both assayed and the result revealed that the asymmetrical analogues are more effective (IC_{50} < 1000 nM) as NQO1 inhibitors compared to symmetrical series (IC_{50} ≥ 1500 nM) as illustrated in Table 1. Based on computational docking experiments conducted by Stratford et al., (2009), they proposed that the NQO1 inhibitors must be capable of hydrogen bonding interactions with the FAD cofactor and/ or the key amino acid residues within the active site (Tyr-126, Tyr-128 and His-161).

The bulky substituted phenyl groups in the symmetrical series would however, undergo steric clashes with the key amino acid residues especially against the hydrophobic pocket which forms the internal wall of the binding site or FAD pocket. It is also observed that the asymmetrical analogues bearing a substituted naphthyl ring displayed higher inhibitory potency than those with a substituted phenyl ring. These differences in the IC_{50} values could be as a result of the naphthyl ring undergoing hydrophobic interactions with the NQO1 enzyme. The phenyl ring is less hydrophobic than the naphthyl ring and this may be the reason why the analogues bearing the phenyl ring are less potent as NQO1 inhibitors. In order to increase the hydrophobic nature of (**2c**), compound (**2e**) was synthesized. Significantly, the IC_{50} improves from 341 ± 115 nM to 85 ± 49 nM. This result suggested that the NQO1 active site binds more effectively to hydrophobic compounds.

Since the use of cytotoxic drugs still remain an unavoidable therapeutic approach to the treatment of malignant tumors, cytotoxicity assay was carried out. Interestingly, only compound (**2d**) displayed toxicity (IC_{50} = 9.2 ± 0.3 μM) towards the A549 cancer cell line while (**2a**, **2b**, **2c** and **2e**) were inactive. The reason for this significant difference is still unknown.

Table 1. IC_{50} values of the asymmetrical analogues of dicoumarol (2a-f).

Entry	Y	**NQO1, IC_{50} (nM)**
2a	2-hydroxyphenyl	165 ± 88
2b	2-hydroxy-1-naphthyl	25 ± 10
2c	2-hydroxyphenyl	341 ± 115
2d	2-hydroxy-1-naphthyl	20 ± 6
2e	2-hydroxyphenyl	85 ± 49
2f	2-hydroxy-1-naphthyl	23 ± 5

4. Experimental

4.1 General

A Biotage Initiator TM microwave reactor (maximum power output of 300 W; operating frequency 2450 MHz) was used. Melting point was measured using a Sanyo Gallenkamp MPD.350 variable heater instrument and are uncorrected. IR spectra were recorded in the solid state using a Bruker Alpha P FT-IR instrument. ^{1}H-NMR spectra were recorded using Bruker Avance 500 spectrometers. Chemical shifts are given in ppm to the nearest 0.01 ppm and referenced to the

solvent residual peak. The coupling constant (*J*) are given in Hz. The abbreviations used are s-singlet, d-doublet, t-triplet, dd-doublet of doublets, td-triplet of doublets, m-multiplet.

General method for the synthesis 4-hydroxycoumarin derivatives (**4a** and **4b**).

Method A: An appropriate acetophenone (500 mg, 3.0 mmol) dissolved in diethyl carbonate (3 mL) was added to a suspension of sodium hydride (60% dispersion in mineral oil, 600 mg, 15.0 mmol) in diethyl carbonate (3 mL) and heated at 100 ^{o}C for 3 hours. The reaction mixture was left to cool to 0 ^{o}C in an ice bath and it was then quenched by dropwise addition of water until effervescence stopped. The aqueous layer was washed with diethyl ether (3 x 10 mL). Concentrated HCl was added dropwise to the aqueous layer to adjust the pH to 4 and the resulting precipitate was collected by filtration, washed with water and left to dry overnight at 90 ^{o}C.

Synthesis of 4-hydroxy-5-methoxy-2H-chromen-2-one (**4a**).

Using 2-hydroxy-6-methoxyacetophenone, compound (**4a**) was isolated as an off-white solid (360 mg, 62%). Mp 155-157 ^{o}C; V_{max}/Cm^{-1} 3260 (w, OH), 1705 (s, C=O), 1640n(s, C=C); δ_H (500 MHz; DMSO-d_6) 3.89 (3H, s, OC$\underline{H}_3$), 5.50 (1 H, s,), 6.95 (2H, d, *J* 8.5,) 7.56 (1H, t, *J* 8.5,), 11.35 (1H, s, OH); m/z (+ES) 215.1 ($[M+Na]^+$, 100%); Found 215.03229; $C_{10}H_8O_4Na$ ($[M+Na]^+$), requires 215.0320).

Synthesis of 4-hydroxy-2H-benzo[h]chromen-2-one (**4b**).

Using 1-hydroxy-2-acetophenone, compound (**4b**) was isolated as an off white solid (67% yield): Mp 285-287 ^{o}C; V_{max}/Cm^{-1} 3410 (br, OH), 1644 (s, C=O); δ_H (500 MHz; DMSO-d_6) 5.70 (1H, s, C(3)$\underline{H}$), 7.70-7.74 (2H, m), 7.82-7.84 (2H, m), 8.03-8.05 (1H, m), 8.34-8.36 (1H, m), 12.67 (1H, br, s, OH); m/z (-ES) 211.1 ($[M-H]^-$, 100%); Found 211.0392; $C_{13}H_7O_3$ ($[M-H]^-$), requires 211.0395).

General procedure for the synthesis of symmetrical analogues of dicoumarol (**1a-f**).

Method B: The appropriate 4-hydroxycoumarin (2 equivalents) was reacted with the appropriate aromatic aldehyde (1 equivalent). Ethanol was added to give a solution of 0.5M concentration with respect to 4-hydroxycoumrin. The reaction mixture was subjected to microwave irradiation at 80 ^{o}C for 30 minutes. The resulting mixture was allowed to cool and the precipitate formed collected by filteration, washed with methanol and dried.

General procedure for the synthesis of asymmetrical analogues of dicoumarol (**2a-f**).

Method C: The appropriate 4-hydroxycoumarin (1 equivalent) was reacted with the appropriate aromatic aldehyde (1 equivalent). Ethanol was added to give a solution of 0.5M concentration with respect to 4-hydroxycoumrin. The reaction mixture was subjected to microwave irradiation at 80 ^{o}C for 30 minutes. The resulting mixture was allowed to cool and the precipitate formed collected by filtration, washed with methanol and dried.

Synthesis of 3,3′-(phenyl)methylene)bis(4-hydroxy-2H-chromen-2-one (**1a**).

Using method B, the reaction of 4-hydroxycoumarin (250 mg, 1.50 mmol) and benzaldehyde (159 mg, 0.75 mmol) in ethanol gave the title compound (**1a**) as a white solid (242 mg, 78%): Mp 210-212 ^{o}C; ν_{max}/cm^{-1} 2700 (br,w, OH), 1670 (s, C=O), 1600 (s, C=C); δ_H (500 MHz; DMSO-d_6) 6.3 (1H, s), 7.10-7.12 (3H, m), 7.12-7.21 (2H, m), 7.25-7.32 (4H, m), 7.55 (2H, ddd, *J* 8.2, 7.1, 1.6), 7.85 (2H, dd, *J* 8.2, 1.6); m/z (-ES) 411 ($[M-H]^-$, 100%); Found 435.0856; $C_{25}H_{16}O_6Na$ ($[M+Na]^+$), requires 435.0845).

Synthesis of 3,3′-((4-hydroxyphenyl)methylene)bis(4-hydroxy-2H-chromen-2-one (**1b**).

Using method B, the reaction of 4-hydroxycoumarin (243 mg, 1,50 mmol) and 4-hydroxybenzaldehyde (92 mg, 0.75 mmol) in ethanol gave the title compound (**1b**) as a pale yellow solid (210 mg, 65%): Mp 222-224; ν_{max}/cm^{-1} 3430 (br, OH), 1660 (s, C=O), 1600 (s, C=C); δ_H (500 MHz; DMSO-d_6) 6.19 (1H, s), 6.59 (2H, d, *J* 8.6), 6.89 (2H, dd), 7.25-7.31 (4H, m), 7.54 (2H, ddd, *J* 8.6, 7.1, 1.0), 7.84 (2H, dd, *J* 8.6, 1.0), m/z (+ES) 429 ($[M+H]^+$, 100%); Found 429.0979; $C_{25}H_{17}O_7$ ($[M+H]^+$), requires 429.0974).

Synthesis of 3,3′-([1,1′-bipheyl]-4-ylmethylene)bis(4-hydroxy-2H-chromen-2-one (**1c**).

Using method B, the reaction of 4-hydroxycoumarin (300 mg, 1.85 mmol) and 4-hydroxybenzaldehyde (169 mg, 0.93 mmol) in ethanol gave the title compound (**1c**) as a white solid (380 mg, 84%): Mp 228-230; ν_{max}/cm^{-1} 3330 (br, OH), 1660 (s, C=O), 1600 (s, C=C); δ_H (500 MHz; DMSO-d_6) 6.19 (1H, s), 6.36 (1H, s), 7.21 (2H, d, *J* 8.6), 7.28-7.35 (5H, m), 7.43 (2H, t, *J* 7.7), 7.50 (2H, d, *J* 8.6), 7.62-7.66 (4H, m), 7.88 (2H, dd, *J* 8.6, 1.5); m/z (-ES) 487.1 ($[M-H]^-$, 100%); Found 487.1193; $C_{31}H_{19}O_6$ ($[M-H]^-$), requires 487.1182).

Synthesis of 3,3′-([1,1′-biphenyl]-4-ylmethylene)bis(4-hydroxy-5-methoxy-2H-chromen-2-one (**1d**).

Using method B, the reaction of 4-hydroxy-6-methoxycoumarin (121 mg, 0.63 mmol) and 4-phenylbenzaldehyde (57 mg, 0.32 mmol) in ethanol gave the title compound (**1d**) as a white solid (57 mg, 66%): Mp 285-287 ^{o}C; ν_{max}/cm^{-1} 3320

(br, OH), 1690 (s, C=O), 1640 (s, C=C); δ_H (500 MHz; $CDCl_3$) 4.01 (6H, s), 6.30 (1H, s), 6.78 (2H, d, *J* 8.4), 7.04 (2H, d, *J* 8.4), 7.37-7.43 (4H, m), 7.46 (2H, t, *J* 8.4), 7.51 (2H, d, *J* 8.4), 7.59 (2H, d, *J* 8.4), 10.21 (2H, s); m/z (+ES) 549 ($[M+H]^+$, 100%); Found 571.1345; $C_{33}H_{24}O_8$ ($[M+Na]^+$), requires 571.1369).

Synthesis of 3,3′-([1,1′-biphenyl]-4-ylmethylene)bis(4-hydroxy-2H-benzo[h]chromen-2-one (**1e**).

Using method B, the reaction of 4-hydroxy-2H-benzo[h]chromen-2-one (124 mg, 0.56 mmol) and 4-phenylbenzaldehyde (53 mg, 0.29 mmol) in ethanol gave the title compound (**1d**) as a white solid (70 mg, 41%): Mp 257-259°C; ν_{max}/cm^{-1} 3020 (br, w, OH), 1670 (s, C=O), 1630 (s, C=C); δ_H (500 MHz; DMSO-d_6) 6.46 (1H, s), 6.30 (1H, s), 7.28 (3H, m), 7.42 (2H, t, *J* 7.7), 7.51 (2H, d, *J* 8.6), 7.62 (2H, d, *J* 8.6), 7.66-7.71 (4H, m), 7.78 (2H, d, *J* 8.6), 7.93 (2H, d, *J* 8.6), 8.01-8.03 (2H, m), 8.38-8.41 (2H, m); *m/z* (-ES) 587.2 ($[M-H]^-$, 100%); Found 589.1657; $C_{39}H_{25}O_6Na$ ($[M+Na]^+$), requires 589.1651).

Synthesis of 3,3′-((4-fluorophenyl)methylene)bis(4-hydroxy-2H-chromen-2-one (**1f**).

Using method B, the reaction of 4-hydroxycoumarin (200 mg, 1.2 mmol) and 4-fluorobenzaldehyde (74 mg, 0.6 mmol) in ethanol gave the title compound (**1b**) as a white solid (214 mg, 83%): Mp 219-221°C; ν_{max}/cm^{-1} 3430 (br, OH), 1660 (s, C=O), 1600 (s, C=C); δ_H (500 MHz; DMSO-d_6) 6.19 (1H, s), 6.59 (2H, d, *J* 8.6), 6.89 (2H, dd), 7.25-7.31 (4H, m), 7.54 (2H, ddd, *J* 8.6, 7.1, 1.0), 7.84 (2H, dd, *J* 8.6, 1.0), m/z (+ES) 429 ($[M+H]^+$, 100%); Found 429.0979; $C_{25}H_{17}O_7$ ($[M+H]^+$), requires 429.0974).

Synthesis of (*E*)-3-(2-hydroxybenzylidiene)-5-methoxychroman-2,4-dione (**2a**).

Using method C, reaction of 4-hydroxy-5-methoxy-2H-chromen-2-one (65 mg, 0.34 mmol) and 2-hydroxybenzyaldehyde (41 mg, 0.34 mmol) gave the title compound (**2d**) as a yellow foam (31 mg, 30%): Mp 178-180°C; ν_{max}/cm^{-1} 3050 (w, OH), 1720 (s, C=O), 1600 (s, C=C); δ_H (500 MHz; $CDCl_3$) 3.68 (3H, s), 6.37-6.39 (1H, m), 6.66 (1H, dd, *J* 8.6, 0.8), 7.33-7.45 (3H, m), 7.59-7.63 (2H, m), 7.88 (1H, s), 12.07 (1H, s); *m/z* (+ES) 319.1 ($[M+Na]^+$, 100%); Found 319.0579; $C_{17}H_{12}O_5Na$ ($[M+Na]^+$), requires 319.0582.

Synthesis of (*E*)-3-((2-hydroxynaphthalen-1-yl)methylene)-5-methoxychroman-2,4-dione (**2b**).

Using method C, reaction of 4-hydroxy-5-methoxy-2H-chromen-2-one (60 mg, 0.31 mmol) and 2-hydroxy-1-naphthaldehyde (50 mg, 0.31 mmol) gave the title compound (**2c**) as a yellow foam (30 mg, 28%): Mp 234-236°C; ν_{max}/cm^{-1} 3075 (w, OH), 1730 (s, C=O), 1560 (s, C=C); δ_H (500 MHz; $CDCl_3$) 3.69 (3H, s), 6.40 (1H, d, *J* 8.3), 6.55-6.60 (1H, m), 7.44 (1H, t, *J* 8.1), 7.53 (1H, d, *J* 8.3), 7.60-7.64 (1H, m), 7.75 (1H, ddd, *J* 8.3, 7.2, 1.0), 7.96 (1H, d, *J* 8.1), 8.08 (1H, d, *J* 8.1), 8.34 (1H, d, *J* 8.3), 8.71 (1H, s), 12.03 (1H, s); *m/z* (+ES) 369.1 ($[M+Na]^+$, 100%); Found 369.0751; $C_{21}H_{14}O_5Na$ ($[M+Na]^+$), requires 369.0739.

Synthesis of (*E*)-3-(2-hydroxbenzylidene)chroman-2,4-dione (**2c**).

Using method C, the reaction of 4-hydroxycoumarin (540 mg, 3.33 mmol) and 2-hydroxy benzyaldehyde (407 mg, 3.33 mmol) in ethanol gave the title compound (**2a**) as a yellow foam (250 mg, 28%): Mp 171-171-173°C; ν_{max}/cm^{-1} 3300 (br, OH), 1720 (s, C=O), 1630 (s, C=C); δ_H (500 MHz; $CDCl_3$), 6.91 (1H ddd, *J* 8.0, 7.2, 1.1), 7.07-7.09 (1H, m), 7.39 (1H, ddd, *J* 8.0, 7.2, 1.1), 7.43-7.45 (1H, m), 7.53-7.57 (2H, m), 7.60 (1H, dd, *J* 8.0, 1.6), 7.68 (1H, ddd, *J* 8.0, 7.2, 1.6), 7.98 (1H, s), 11.75 (1H, s); *m/z* (+ES) 289.05 ($[M+Na]^+$, 100%); Found 289.0477; $C_{16}H_{10}O_4Na$ ($[M+Na]^+$), requires 289.0475).

Synthesis of (*E*)-3-((2-hydroxynaphthalen-1-yl)methylene)chroman-2,4-dione (**2d**).

Using method C, reaction of 4-hydroxycoumarin (500 mg, 3.08 mmol) and 2-hydroxy-1-naphthaldehyde (530 mg, 3.08 mmol) gave the title compound (**2b**) as a yellow solid (300 mg, 31%): Mp 247-249°C; ν_{max}/cm^{-1} 3080 (br, w, OH), 1710 (s, C=O), 1570 (s, C=C); δ_H (500 MHz; DMSO-d_6) 6.94-6.98 (2H, m), 7.48-7.53 (1H, m), 7.64-7.68 (2H, m), 7.74-7.78 (2H, m), 8.11 (1H, *d*, J 7.8), 8.31(1H, *d*, J 7.8), 8.64 (1H, d, *J* 8.3), 9.12 (1H, s), 10.78 (1H, s); *m/z* (+ES) 339.0 ($[M+Na]^+$, 100%); Found 339.0633; $C_{20}H_{12}O_4Na$ ($[M+Na]^+$), requires 339.0626.

Synthesis of (*E*)-3-(2-hydroxybenzylidiene)-2H-benzo[h]chromen-2,4(3H)-dione (**2e**).

Using method C, reaction of 4-hydroxy-2H-benzo[h]chromen-2-one (300 mg, 1.42 mmol) and 2-hydroxybenzyaldehyde (150 mg, 1.42 mmol) gave the title compound (**2e**) as a yellow foam (140 mg, 31%): Mp 226-228°C; ν_{max}/cm^{-1} 3030 (w, OH), 1720 (s, C=O), 1600 (s, C=C); δ_H (500 MHz; $CDCl_3$) 7.25 (1H, dd, *J* 7.6, 1.4), 7.37-7.39 (1H, m), 7.43-7.46 (2H, m), 7.56-7.59 (1H, m), 7.63 (1H, dd, J 7.6, 1.4), 7.66-7.70 (2H, m), 7.77 (1H, d, *J* 8.2), 8.04 (1H, s), 8.52 (1H, d, *J* 8.2), 13.59 (1H, s); *m/z* (+ES) 317.0 ($[M+H]^+$, 100%); Found 339.0645; $C_{20}H_{12}O_4Na$ ($[M+Na]^+$), requires 339.0633.

Synthesis of (*E*)-3-((2-hydroxynaphthalen-1-yl)methylene)-H-benzo[h]chromen-2,4(3H)-dione (**2f**).

Using method C, reaction of 4-hydroxy-2H-benzo[h]chromen-2-one (200 mg, 0.94 mmol) and

2-hydroxy-1-naphthaldehyde (162 mg, 0.94 mmol) gave the title compound (**2f**) as orange foam (90 mg, 26%): Mp 250-252 ^{o}C; ν_{max}/cm^{-1} 3060 (m, OH), 1720 (s, C=O), 1600 (s, C=C); δ_H (500 MHz; $CDCl_3$) 7.51 (1H, d, *J* 9.0), 7.56-7.60 (3H, m), 7.64 (1H, ddd, *J* 8.2, 7.0, 1.4), 7.69 (1H, ddd, *J* 8.2, 7.0, 1.4), 7.75 (1H, ddd, *J* 8.2, 7.0, 1.4), 7.78 (1H, d, *J* 8.1), 7.99 (1H, d, *J* 8.1), 8.15 (1H, d, *J* 9.0), 8.28 (1H, d, *J* 8.2), 8,54 (1H, d, *J* 8.2), 8.86 (1H, s), 13.67 (1H, s); *m/z* (+ES) 367.0 ($[M+H]^+$, 80%); Found 389.0794; $C_{24}H_{14}O_4Na$ ($[M+Na]^+$), requires 389.0790.

5. Enzyme Assays

Serial dilution of the stock solutions of the synthetic dicoumarol analogues (10 mM concentration) were prepared in six cuvettes using DMSO to give concentration ranging from 100 nM to 1 mM (0.1 µM – 1000 µM). NQO1 enzyme was diluted in 50 mM phosphate buffer to give an enzyme activity within the 0.085-0.14 nM range. The IC_{50} values were measured using nonlinear fitting as implemented in the program Excel (GraphPad Prism 5). Each measurement was made in triplicate and the experiments were repeated three times. The concentration-response plots obtained displayed a sigmoid response curve (Figure 3), and demonstrated moderate to good inhibitory potency. Low values indicate that the inhibitors have good inhibitory potency, while the high values indicated poor inhibition.

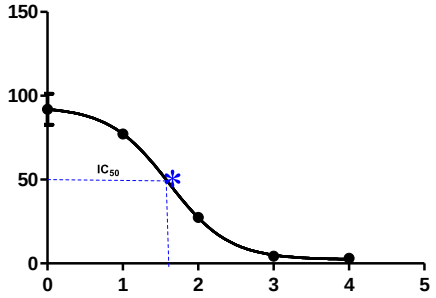


Figure 3. A sigmoid curve for the concentration-response plot of the enzyme assay

6. MTT Cell Viability Assay

The A549 cell line was obtained from the American Type Culture Collection (ATCC) and grown in RPMI 1640 medium with 10% fetal bovin serum. Cells were seeded in 96-well flatbottom microtiter plates and then allowed to attach overnight (24 hours) at 37 ^{o}C with a 5% CO_2 in air humidified environment before the drug treatment.

7. Conclusion

In summary, all the synthesized asymmetrical analogues of dicoumarol (**2a-f**) showed moderate inhibitory potency towards NQO1 with the exception of compound (**2c**) due to hydrophobic nature of the NQO1 active site. Compound (**2d**) in particular displayed good toxicity towards A549 small cancer cell line and thus might be promising for the development of new antitumor agents.

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