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Functionalized 3-benzylidene-indolin-2-ones: Inducers of NAD(P)H-quinone oxidoreductase 1 (NQO1) with antiproliferative activity

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ABSTRACT

Functionalized benzylidene-indolin-2-ones are widely associated with antiproliferative activity. The scaffold is not normally associated with chemoprevention in spite of the presence of a nitrogen-linked Michael acceptor moiety that may predispose members to induction of NQO1, a widely used biomarker of chemopreventive potential. To investigate this possibility, we have synthesized and evaluated a series of functionalized 3-benzylidene-indolin-2-ones for induction of NQO1 in murine Hepa1c1c7 cells as well as antiproliferative activity against two human cancer cell lines (MCF-7, HCT116). The benzylideneindolinones were found to be good inducers of NQO1 activity, with 85% of test compounds able to increase basal NQO1 activity by more than twofold at concentrations of $\leqslant$10 μM. By contrast, fewer compounds (11%) tested at the same concentration were able to reduce cell viability by more than 50%. Structure activity relationships showed that the nitrogen linked Michael acceptor moiety was an essential requirement for both activities. This common feature notwithstanding, substitution of the 3-benzylidene-indolin-2-one core structure affected NQO1 induction and antiproliferative activities in dissimilar ways, underscoring different structural requirements for these two activities. Nonetheless, promising compounds (**10**, **42**, **45**–**48**) were identified that combine selective induction of NQO1 with potent antiproliferative activity. A potential advantage of such agents would be the ability to provide added protection to normal cells by the up-regulation of NQO1 and other phase II enzymes while simultaneously targeting neoplastic cells.

1. Introduction

Carcinogenesis is a complex and protracted multistep process involving distinct yet closely linked events of tumour initiation, promotion and progression.[1,2] The multi-stage nature of carcinogenesis has led to the proposal that timely intervention with dietary or pharmaceutical agents may result in the prevention, delay or reversal of the process. This concept is termed cancer chemoprevention and was espoused by Sporn et al. as a strategy for reducing cancer mortality.[3] Chemopreventive agents may block or reverse the premalignant stages of initiation and promotion. They may also retard the transformation of precancerous cells to malignant cells. The mechanisms of chemoprevention are complex and involve multiple signaling transduction pathways.[4–6]

The enzyme NAD(P)H: quinone oxidoreductase 1 (NQO1) is widely recognized for its role in protecting cells against the toxic effects of pro-oxidant chemicals. In the presence of oxidative stress, expression of NQO1 is increased to provide timely protection of the cell by various mechanisms such as the reduction of exogenous quinones to quinols, thus by-passing the formation of reactive and toxic semiquinone intermediates[7,8] and an indirect antioxidant effect which maintains endogenous antioxidants in their reduced/active states.[9–11] The induction of NQO1 is associated with the up-regulation of other protective Phase II enzymes.[12,13] Since the measurement of NQO1 induction is a well established procedure with medium-throughput capabilities, it is widely employed as a biomarker to screen compounds for chemopreventive potential against cancer initiation.[14–16] NQO1 is also up-regulated in many tumours and recent reports that it can stabilize the tumour suppressor protein p53 either by inhibiting its degradation or by a direct protein–protein interaction has heightened interest in the enzyme.[17,18] Observations that NQO1-deficient mice had lower levels of p53 induction, reduced apoptosis, increased susceptibility to chemically induced tumours and impaired NF-κB function have further underscored its importance in carcinogenesis.[19–21] With this background in mind, the focus of our investigations is to develop compounds with chemopreventive potential based on their ability to induce NQO1 activity in cell-based assays.

The seminal work of Talalay and co-workers has led to the recognition of the Michael acceptor motif as a recurrent structural entity in compounds with chemopreventive activity.[22–24] Cinnamates, chalcones, curcuminoids, coumarins, phenylbutenones,

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bis(benzylidene) cycloalkanones and triterpenoids are relevant examples.[22,23,25] In the present study, we propose that functionalized 3-benzylidene-indolin-2-ones should likewise be included as compounds with chemopreventive potential. The presence of the Michael acceptor motif makes the benzylideneindolinone scaffold a likely candidate for such activity. One difference is that the exocyclic double bond in this template is linked to an amide carbonyl, and not the normal carbon-linked carbonyl found in previously reported compounds (Fig. 1a). Thus the motif is better described as a 'nitrogen-linked Michael acceptor'. Since little is known of the chemopreventive properties of such an entity, investigations on 3-benzylidene-indolin-2-ones would provide timely insight.

Arylideneindolinones are widely reputed to be anti-tumour agents.[26,27] A representative member of this class is sunitinib which is currently used in the clinics as a multi-targeting tyrosine kinase inhibitor with antiangiogenic activity (Fig. 1b). If antitumour benzylideneindolinones have chemopreventive properties, this may mean that when deployed for their effects on cancer cells, the same agent would confer cytoprotection to normal/non-metastasized cells, an interesting proposition with potential clinical benefits. It is thus appropriate to determine if there is any overlap in antiproliferative and chemopreventive properties in the benzylideneindolinone scaffold, and if so, to establish structure–activity relationships for future lead optimization.

Our interest in the Michael acceptor-like motif in 3-benzyliden-indolin-2-ones is also motivated by the mechanistic aspects of NQO1 induction. Briefly, induction of NQO1 gene expression is controlled by the arylhydrocarbon (AhR)/xenobiotic response element (XRE) and the Kelch-like ECH-associated protein 1 (Keap1)/nuclear factor erythroid 2-related factor (Nrf2)/antioxidant response element (ARE) signaling pathways. Compounds that induce NQO1 by stimulating the AhR/XRE pathway are described as bi-functional inducing agents.[28] By contrast, compounds that transcriptionally activate the NQO1 gene through the ARE pathway are designated mono-functional inducers. Bi-functional inducers are AhR agonists and induce both Phase I (CYP1A1, 1A2, 1B1) and Phase II (including NQO1, GSTA2, UGT1A6) enzymes.[29,30] Mono-functional inducers are disruptors of the Keap1/Nrf2 complex. The liberated Nrf2 migrates to the nucleus where it binds to the ARE enhancer regions to bring about transcription of Phase II enzymes.[24] It has been argued that induction of Phase I enzymes is potentially deleterious to the cell because CYP450 can activate polycyclic aromatic hydrocarbons to form ultimate carcinogens. Thus mono-functional inducers are deemed more desirable than bi-functional inducers.[2,16] But this may be an oversimplification of a complex issue, in view of emerging evidence of cross-talk between the AhR and Nrf2 gene batteries.[6,31]

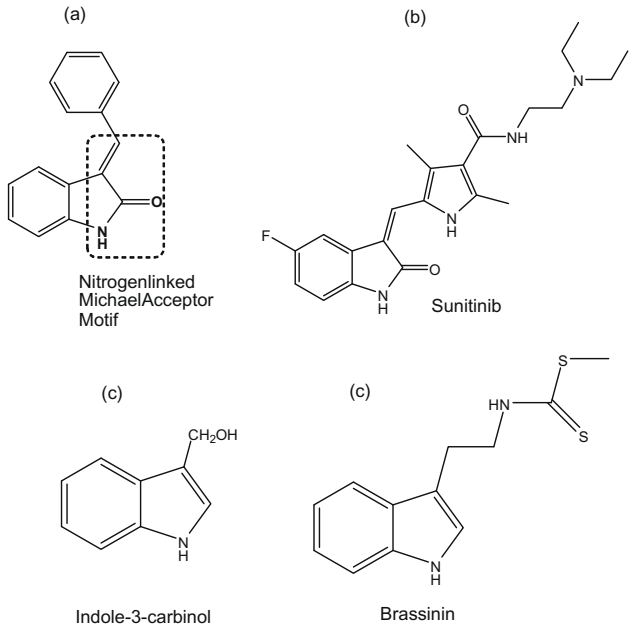


Figure 1. (a) Nitrogen linked Michael acceptor moiety in benzylideneindolinones; (b) structure of sunitinib; (c) structures of indole-3-carbinol and brassinin.

Compounds with Michael acceptor motifs are associated with mono-functional induction of NQO1.[22–24] On the other hand, indole phytochemicals like indole-3-carbinol and brassinin are bi-functional inducers (Fig. 1c).[32,33] Since the 3-benzylideneindolin-2-one template embraces both structural elements—the indole ring and the embedded Michael acceptor moiety, this raises the question as to whether bi- or mono-functional induction would prevail in these compounds. To address these issues, we have synthesized a series of functionalized 3-benzylidene-indolin-2-ones and related compounds and evaluated their chemopreventive potential in terms of the induction of NQO1 activity in Hepa1c1c7 cells. The compounds were also evaluated for induction of CYP1A1 activity on the same cell line to determine if there is concurrent induction of CYP1A1 activity and if so, the extent to which CYP1A1 is induced compared to NQO1. Finally, the compounds were screened for antiproliferative activity on two cancer cell lines with the aim of assessing the extent to which both properties are found in the benzylideneindolinone scaffold.

2. Results

2.1. Synthesis of target compounds

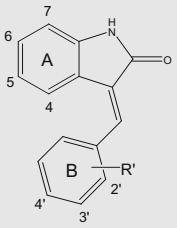
The synthesized compounds were divided into four classes based on their structural features. Class 1 comprised 3-benzylideneindolin-2-ones with different substituents on the benzylidene ring B. The choice of substituents was guided by the Craig Plot to ensure that groups of different lipophilicites and electronic effects were adequately represented.[34] Ring B was also replaced by heterocycles (pyridine, indole) or naphthalene. Class 2 compounds were substituted on both the indole ring A and the benzylidene ring B. The groups on ring B were 3′-OCH_3, 3′-OH-4′-OCH_3 or 3′-CF_3. For each ring B substituent, three different ring A groups were explored (F, Cl, OCH_3). Class 3 compounds were structural variants of Class 1 compounds and consisted of the following modifications: (i) N-methylation of the indolinone nitrogen (**49**, **50**); (ii) reduction of the exocyclic double bond (**51**, **52**) and (iii) shift in the positions of the carbonyl and exocyclic double bond to give the regioisomeric 2-benzylidene-indolin-3-ones (**53**, **54**). Class 4 consisted of isoindigos (3,3′-bisindoles) which may be viewed as two indolin-2-ones joined by an exocyclic double bond and with two nitrogen-linked Michael acceptor moieties in the scaffold. The structures of Class 1–4 compounds are given in Table 1. Of the 61 compounds listed in this Table, 29 members (**1**–**6**, **8**, **9**, **11**, **12**, **16**, **17**, **20**, **24**, **26**–**28**, **33**–**36**, **42**, **47**, **51**–**53**, **55**, **57**, **58**) have been reported in the literature[27,35–48] (see Supplementary data for exact citations). Except for compound **55**,[49] none have been evaluated for chemopreventive activity based on the induction of NQO1.

The Class 1 and 2 compounds (**1**–**48**) were synthesized by a Knoevenagel reaction between the aldehyde and oxindole. The N-methylated 3-benzylidieneindolin-2-ones **49**, **50** were obtained in a similar way starting from *N*-methyloxindole. The condensation was carried out in ethanol with piperidine as base catalyst by reflux or in a microwave reactor (Scheme 1). The 2-benzylindolin-2-ones **51** and **52** were synthesized by catalytic hydrogenation of the exocyclic double bond of the benzylidineindolin-2-ones **41**

and **20**, respectively. Reduction of **41** was accompanied by the loss of the 6-chloro group to give **51**.

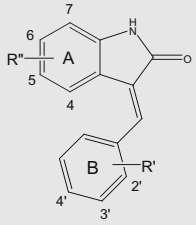
Table 1
Structures of compounds in Class 1–4

(A) Class 1 compounds (**1**–**38**)



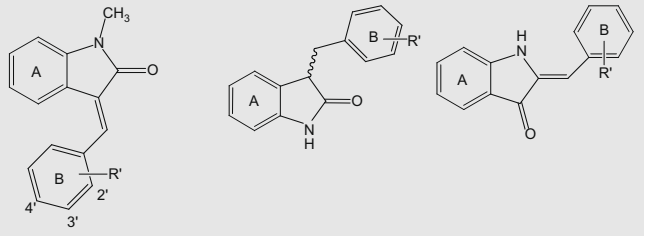
Compound	R′	Compound	R′
1	H	**20**	4′-CH_3
2	2′-OCH_3	**21**	4′-C $_2H_5$
3	3′-OCH_3	**22**	2′-F
4	4′-OCH_3	**23**	3′-F
5	2′,4′-$(OCH_3)_2$	**24**	4′-F
6	2′,5′-$(OCH_3)_2$	**25**	2′,4′-$(F)_2$
7	2′,6′-$(OCH_3)_2$	**26**	3′-Cl
8	3′,5′-$(OCH_3)_2$	**27**	4′-Cl
9	3′,4′,5′-$(OCH_3)_3$	**28**	2′,4′-$(Cl)_2$
10	3′-O-C_6H_5	**29**	2′-Cl, 4′-F
11	2′-OH	**30**	2′-CF_3
12	3′-OH	**31**	3′-CF_3
13	2′,5′-$(OH)_2$	**32**	4′-CF_3
14	3′,5′-$(OH)_2$	**33**	4′-CN
15	2′-OH, 4′-OCH_3	**34**[a]	2′-Pyridinyl
16	3′-OH, 4′-OCH_3	**35**[a]	4′-Pyridinyl
17	3′-OCH_3, 4′-OH	**36**[a]	3′-Indolyl
18	2′-CH_3	**37**[a]	1′-Napthalenyl
19	3′-CH_3	**38**[a]	2′-Napthalenyl

(B) Class 2 compounds (**39**–**48**)



Compound	R′	R″	Compound	R′	R″
39	3′-OCH_3	6-F	**44**	3′-OH, 4′-OCH_3	6-Cl
40	3′-OCH_3	5-Cl	**45**	3′-OH, 4′-OCH_3	6-OCH_3
41	3′-OCH_3	6-Cl	**46**	3′-CF_3	6-F
42	3′-OCH_3	6-OCH_3	**47**	3′-CF_3	6-Cl
43	3′-OH, 4′-OCH_3	6-F	**48**	3′-CF_3	6-OCH_3

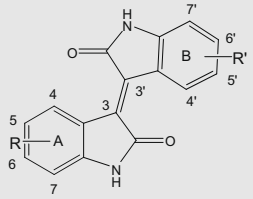
(C) Class 3 compounds: 3-Benzylidene-1-methylindolin-2-ones (**49**,**50**), 3-benzylindolin-2-ones (**51**,**52**) and 2-benzylidene-indolin-3-ones (**53**,**54**)



49: R′ = 3′-OCH_3 **51**: R′ = 3′-OCH_3 **53**: R′ = 3′-OCH_3
50: R′ = 3′-OH, 4′-OCH_3 **52**: R′ = 4′-CH_3 **54**: R′ = 3′-OH, 4′-OCH_3

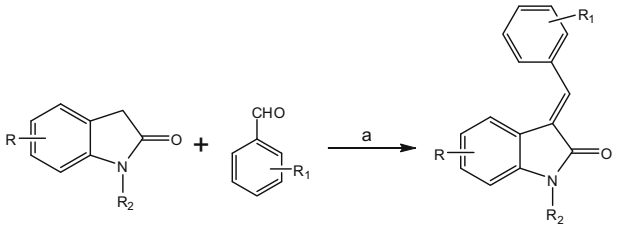
Table 1 (*continued*)

(D) Class 4 compounds (**55**–**61**)

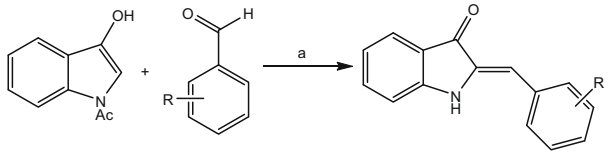


Compound	R	R′	Compound	R	R′
55	H	H	**59**	6-CF_3	H
56	6-F	H	**60**	6-CF_3	5-F
57	5-Cl	H	**61**	6-CF_3	5-Cl
58	6-Cl	H			

[a] Ring B is replaced by pyridine, indole or naphthalene.



Scheme 1. Synthetic pathway for Class 1 and 2 compounds (**1**–**48**). Reagents and conditions: (a) piperidine (cat.), ethanol, microwave, 140 °C, 15 min or reflux, 18 h. R_2 = Methyl for **49** and **50**. Substituted benzaldehydes replaced by appropriate carbaldehydes for **34**–**38**.

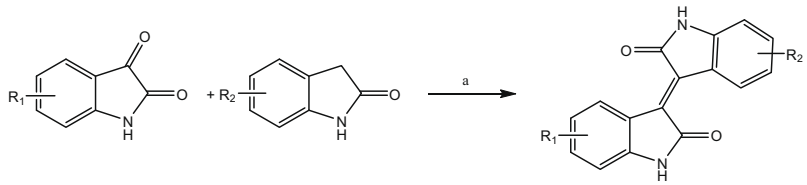


Scheme 2. Synthetic pathway for substituted 2-benzylidene indolin-3-ones (**53**, **54**). Reagents and conditions: (a) 50% aqueous ethanol, concentrated hydrochloric acid, reflux 2 h.

The 2-benzylidineindolin-3-ones (**53**, **54**) were obtained by an acid catalyzed aldol condensation (Scheme 2). The presence of acid promoted the loss of the *N*-acetyl group from 1-(3-hydroxy-1*H*-indol-1-yl) ethanone and generated a carbocation in the reacting aldehyde. Reaction between the α carbon of 1*H*-indol-3-ol and the carbocation proceeded with loss of water to yield the desired product. The isoindigos (**55**–**61**) were obtained by an acid catalyzed reaction between an oxindole and isatin (Scheme 3). The mechanism is also an acid catalyzed aldol condensation and involved enolization of the oxindole, protonation of the non-amide carbonyl oxygen of the isatin, followed by condensation and dehydration to give the desired isoindigo.

2.2. Assignment of configuration of synthesized compounds

The synthesized compounds (except **51**, **52**) could exist as either *E* or *Z* isomers due to the presence of the exocyclic double bond. Thus it was necessary to determine if synthesis had given



Scheme 3. Synthetic pathway for isoindigos (**55**–**61**). Reagents and conditions: (a) glacial acetic acid, concentrated hydrochloric acid, reflux 16 h.

rise to a single (predominant) isomer or to a mixture of isomers. Analyses of the ^{1}H and ^{13}C NMR spectra of the Class 1–3 compounds (except **51**, **52**) showed that they were obtained as a single (or predominant) isomer.

To determine if the Class 1–2 compounds were *E* or *Z* isomers, the chemical shifts of the aromatic protons on the benzylidene ring B (H-2′ and H-6′) were analyzed. In the *Z* but not *E* isomer, the H-2′ or H-6′ protons would be shifted downfield due to deshielding by the carbonyl group. In the literature, chemical shifts of 7.85–8.53 ppm were assigned to the *Z* isomer, and 7.45–7.84 ppm to the *E* isomer.[26,27] For compounds already reported to be *E* isomers and whose chemical shifts were available from the literature, comparisons of our experimental values with reported values provided the needed confirmation.[27,36,37,39,40,44–46]

For compounds whose stereochemistries have not been previously assigned, the chemical shifts of the H-2′ and H-6′ protons at 7.45–7.84 ppm were used to conclude that the synthesized compounds were *E* isomers. Compounds **23**, **29**, **32**, **46**, **47** were obtained as a mixture of *E* and *Z* isomers but with one predominant isomer. When the ^{1}H spectra of the isomers (after separation by column chromatography) were examined, the chemical shifts of H-2′ and H-6′ were found at 7.90–8.01 ppm for the predominant isomer, and 8.40–8.50 ppm for the minor isomer. Hence, the predominant isomer was assigned the *E* configuration.

^{1}H chemical shifts could not be used to assign the configuration of **7** as it had methoxy groups on both the 2′ and 6′ positions of ring B. Thus, a 2D NOESY analysis was carried out. If **7** was present as the *Z* isomer, NOESY analysis would reveal interactions between the vinylic proton and H-4 on the indolinone ring. Since the latter interaction was not observed in the spectrum (Supplementary data), it was concluded that **7** was present as the *E* isomer.

In the case of **21** and **37**, X-ray analyses showed that they were present as E isomers (Fig. 2) and this led us to assign the *E* configuration to other 3-(benzylidene) indolin-2-ones with 4′ substituents (**4**, **24**, **27**, **33**), as well as **38** which is a regioisomer of **37**. A check on the ^{1}H chemical shifts of these compounds provided further confirmation.

The Class 3 compounds **53** and **54** are 2-benzylideneindolin-3-ones. **53** was reported to be a *Z* isomer[38] and a comparison of the reported ^{13}C chemical shifts with the experimentally obtained values for **53** showed good agreement. By the same token, **54** was assigned a *Z* configuration based on the similarity of its spectral characteristics (^{13}C and ^{1}H chemical shifts, aromatic region) to **53**.

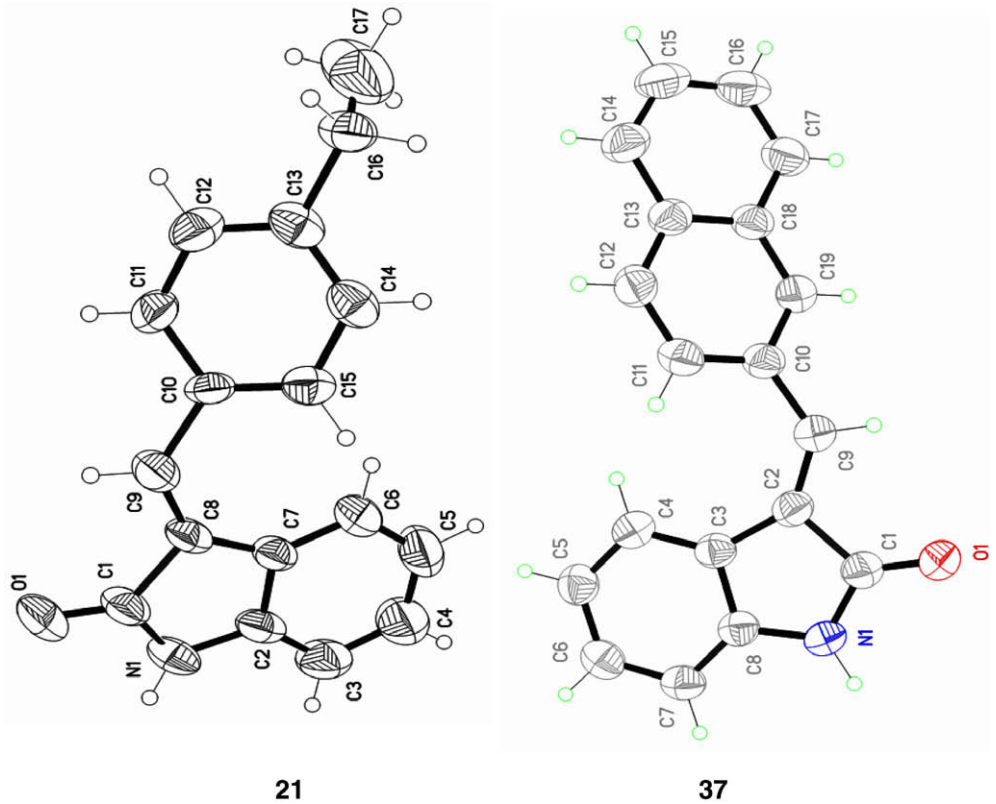


Figure 2. X-ray structures of **21** and **37**.

As for the Class 4 compounds, we found recurrent similarities in the ^{13}C and ^{1}H spectra of all the members (**55**–**61**). Since isoindigo (**55**) was reported to have an *E* configuration,[42] the other isoindigos (**56**–**61**) in Class 4 were likewise assigned as *E* isomers.

A chiral centre at C3 is present in the reduced compounds **51** and **52**, but no attempt was made to determine if they were *R*, S or a racemate. The compounds were purified in the usual way and used as such for biological testing.

Table 2
CD and IC_{50} values of Class 1–4 compounds on Hepa1c1c7 cells

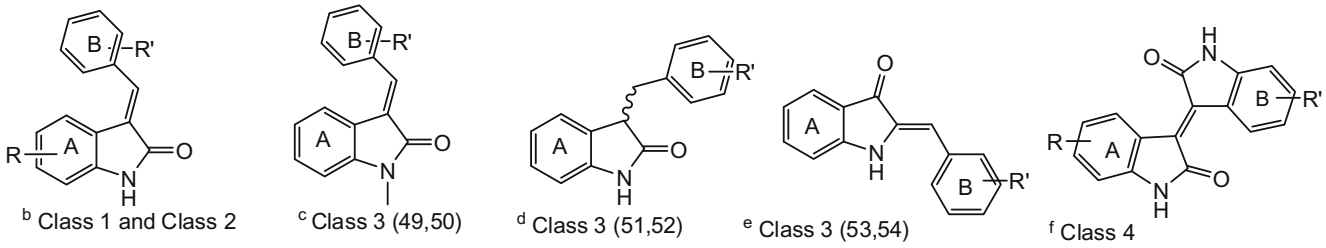
Number	Ring A (R)	Ring B (R′)	QR Induction of Hepa1c1c cells[a]CD (μM)	IC_{50} (μM) on Hepa1c1c7 cells
Class 1[b]				
1	H	H	0.92 ± 0.09	>25
2	H	2′-OCH_3	0.25 ± 0.06	>25
3	H	3′-OCH_3	0.27 ± 0.06	>25
4	H	4′-OCH_3	0.87 ± 0.11	>25
5	H	2′,4′ -$(OCH_3)_2$	>11	11.1
6	H	2′,5′-$(OCH_3)_2$	0.013 ± 0.003	>25
7	H	2′,6′-$(OCH_3)_2$	>14	13.9
8	H	3′,5′-$(OCH_3)_2$	>16	16.0
9	H	3′,4′,5′-$(OCH_3)_3$	>25	>25
10	H	3′ -OC_6H_5	1.7 ± 0.4	10.8
11	H	2′-OH	9.0 ± 1.0	>25
12	H	3′-OH	1.1 ± 0.2	>25
13	H	2′,5′-$(OH)_2$	2.4 ± 0.6	>25
14	H	3′,5′-$(OH)_2$	5.3 ± 0.6	>25
15	H	2′-OH, 4′-OCH_3	0.49 ± 0.13	>25
16	H	3′-OH, 4′-OCH_3	0.12 ± 0.03	11.9
17	H	3′-OCH_3, 4′-OH	0.86 ± 0.08	14.6
18	H	2′CH_3	0.85 ± 0.07	>25
19	H	3′-CH_3	0.27 ± 0.05	>25
20	H	4′-CH_3	1.6 ± 0.2	>25
21	H	4′ -C_2H_5	2.4 ± 0.4	>25
22	H	2′-F	0.85 ± 0.07	>25
23	H	3′-F	1.73 ± 0.4	>25
24	H	4′-F	5.1 ± 0.7	>25
25	H	2′,4′-$(F)_2$	3.3 ± 0.6	>25
26	H	3′-Cl	0.12±0.03	5.9
27	H	4′-Cl	0.45 ± 0.1	6.7
28	H	2′, 4′-$(Cl)_2$	2.3 ± 0.6	10.6
29	H	2′-Cl, 4′-F	0.33±0.08	>25
30	H	2′-CF_3	4.5 ± 0.6	>25
31	H	3′-CF_3	0.027 ± 0.006	3.9
32	H	4′-CF_3	0.031 ± 0.009	12.0
33	H	4′-CN	20.7±2.1	>25
34	H	Ring B = 2′-pyridinyl	2.7 ± 0.5	19.9
35	H	Ring B = 4′-pyridinyl	14.4 ± 1.3	>25
36	H	Ring B = 3′-indolyl	0.17 ± 0.05	>25
37	H	Ring B = 1′-napthalenyl	>19	18.6
38	H	Ring B = 2′-napthalenyl	0.42±0.06	>25
Class 2[b]				
39	6-F	3′-OCH_3	0.17 ± 0.05	9.8
40	5-Cl	3′-OCH_3	0.36 ± 0.06	17.5
41	6-Cl	3′-OCH_3	0.039 ± 0.01	>25
42	6-OCH_3	3′-OCH_3	0.13 ± 0.05	9.8
43	6-F	3′-OH, 4′-OCH_3	0.11 ± 0.05	15.6
44	6-Cl	3′-OH, 4′-OCH_3	0.076 ± 0.01	>25
45	6-OCH_3	3′-OH, 4′-OCH_3	0.24 ± 0.08	9.8
46	6-F	3′-CF_3	0.016 ± 0.007	2.2
47	6-Cl	3′-CF_3	0.0041 ± 0.0009	1.1
48	6-OCH_3	3′-CF_3	0.085 ± 0.014	2.6
Class 3[f,g,h]				
49[c]	—	3′OCH_3	1.07 ± 0.15	>25
50[c]	—	3′-OH, 4′-OCH_3	1.2 ± 0.3	15.5
51[d]	—	3′-OCH_3	10.9 ± 1.1	>25
52[d]	—	4′-CH_3	>25	>25
53[e]	—	3′-OCH_3	0.49 ± 0.07	21.2
54[e]	—	3′-OH, 4′-OCH_3	1.9 ± 0.2	>25
Class 4[f]				
55	H	H	0.025 ± 0.008	>25
56	6-F	H	0.0083 ± 0.0016	>25
57	5-Cl	H	0.0015 ± 0.0007	20.5
58	6-Cl	H	0.0017 ± 0.00097	>25

(*continued on next page*)

Table 2 (*continued*)

Number	Ring A (R)	Ring B (R′)	QR Induction of Hepa1c1c cells[a]CD (μM)	IC_{50} (μM) on Hepa1c1c7 cells
59	6-CF_3	H	0.00056 ± 0.00021	>25
60	6-CF_3	5-F	0.00096 ± 0.00016	>25
61	6-CF_3	6-Cl	0.00069 ± 0.0002	9.8

Structures of Class 1–4 are as follows:



[a] Concentration required to bring about a twofold increase in the NQO1 activity of the treated cells compared to the untreated Hepa1c1c7 cells. Mean and SD ($n = 3$).

2.3. Induction of NQO1 in Hepa1c1c7 cells

The compounds were screened for induction of NQO1 activity in the murine hepatoma (Hepa1c1c7) cells.[14] Briefly, the assay is based on the generation of NADPH when glucose-6-phosphate (G6P) is reduced by G6P dehydrogenase in the presence of its cofactor NADP. NADPH serves as an electron donor for the NQO1 mediated reduction of menadione (a quinone) to menadiol (a diphenol). The latter reduces MTT to formazan (a coloured product) and its formation is monitored in the assay. A compound that induces NQO1 increases the rate at which menadiol is formed, and hence the generation of formazan.

The compounds were first screened for their growth inhibitory IC_{50} values on the Hepa1c1c7 cells to ensure that concentrations used in the induction assay were not cytotoxic to the cells. Compounds that did not affect cell viability at 25 μM (highest concentration tested) were listed as having IC_{50} values exceeding 25 μM. It can be seen from Table 2 shows that ≈80% of the compounds had IC_{50} values $\geqslant$10 μM. Once this was established, the compounds were tested over a 10–100-fold concentration range for induction activity, which was expressed in terms of the CD, defined as the concentration required to bring about a twofold increase in NQO1 activity of treated cells compared to untreated Hepa1c1c7 cells. The results are given in Table 2. Positive controls for this assay were the known NQO1 inducers sulforaphane and β-napthoflavone (BNF). Their CD values were 0.26 (±0.04) μM and 0.028 (±0.003) μM, which agreed well with reported values.[25]

As seen from Table 2, not all compounds had CD values. Notably, **5**, **7**, **8** and **37** had no effect on NQO1 induction at concentrations lower than their $IC_{50\,Hepa1c1c7}$, and could not be tested at concentrations greater than $IC_{50\,Hepa1c1c7}$ because of cytotoxic effects on the cells. Their CD values were recorded as $>IC_{50\,Hepa1c1c7}$ rounded up to the nearest unit. No induction was observed for **9** and **52** when they were tested at 25 μM which was the highest concentration used in the assay. Their CDs were listed as >25 μM in Table 2.

The 3-substituted indolin-2-ones of Class 1 ($n = 38$) showed an impressive 1000-fold variation in induction activity. Since only ring B was modified, it was clear that the nature of this ring was a critical determinant of activity. Ring B was either mono-, di- or tri-substituted or replaced by a non-phenyl ring B. These changes had different effects on induction activity as discussed in the following paragraph.

Among the Class 1 compounds with monosubstituted ring B, good activity was associated with the CF_3, Cl and OCH_3 groups at either at 3′ or 4′ position of ring B. The sequence of activity for 4′-monosubstituted compounds was 4′-CF_3 (**32**, 0.031 μM) > 4′-Cl (**27**, 0.45 μM) > 4′-OCH_3 (**4**, 0.87 μM), H (**1**, 0.92 μM) > 4′-CH_3 (**20**, 1.6 μM) > 4′-C_2H_5 (**21**, 2.4 μM) > 4′-F (**24**, 5.1 μM) > 4′-CN (**33**, 20.7 μM). For 3′-monosubstituted compounds, it was 3′-CF_3 (**31**, 0.027 μM) > 3′-Cl (**26**, 0.12 μM) > 3′-OCH_3 (**3**, 0.27 μM), 3′-CH_3 (**19**, 0.27 μM) > H (**1**, 0.92 μM), 3′-OH (**12**, 1.1 μM) > 3′-OC_6H_5 (**10**, 1.70 μM), 3′-F (**23**, 1.73 μM). When the Hansch π and Hammett σ values of these substituents[50] were analyzed for correlation to CD (expressed as $-\log_{10}$ CD or pCD), induction activity was significantly correlated to σ (Spearman's $\rho = 0.42$, $p = 0.045$, 2 tailed) but not π. Thus, activity was determined primarily by the electron density of ring B and to a lesser extent by its lipophilic character. The positive ρ value indicated a preference for electron withdrawing ($+\sigma$) groups like CF_3 and halogens.

When ring B was di- or tri-substituted, no clear trend was observed. By contrast to the preference for electron withdrawing groups observed for mono-substituted ring B, di-substitution of ring B with two halogens did not improve activity, as seen from a comparison of **28** (2′,4′-Cl, CD 2.3 μM) with **26** (3′-Cl, CD 0.12 μM) and **27** (4′-Cl, CD 0.45 μM), as well as **25** (2′,4′-F, CD 3.3 μM) with **22** (2′-F, CD 0.85 μM) and **23** (3′-F, CD 1.73 μM). In the case of OCH_3, the introduction of two or three such groups on ring B (**5**, **7**, **8**, **9**) caused a marked decrease in induction activity. A notable exception is the di-methoxylated derivative **6** (2′,5′-OCH_3, CD 0.013 μM) which was extremely potent and in fact, the most potent inducer among the Class 1 compounds. Its potency may be related to conversion to an active metabolite (perhaps a quinone) that mediates the induction effect.

When a non-phenyl ring was present as ring B, variable outcomes were observed. An interesting observation was the different induction potencies of the isomeric napthalenyl derivatives (**37**, **38**). The 1′ napthalenyl isomer (**37**, CD > 19 μM) was significantly less active than the 2′-napthalenyl isomer (**38**, CD 0.42 μM). The 2′ and 4′-pyridinyl isomers (**34**, **35**) also showed regioselectivity in their induction properties.

Class 2 comprised of a small group of ten 3-benzylideneindolin-2-ones that were substituted on both rings A and B. They were generally more potent NQO1 inducers than the Class 1 compounds, with CD values ranging from 0.36 μM to 4 nM. To determine if substitution on ring A was instrumental in the improved induction profiles, the following comparisons (analyzed by one-way ANOVA) were made. In the case of **3** (3′-OCH_3 on ring B, no ring A substituent) and **39**–**42** (3′-OCH_3 on ring B, various substituents on ring A), greater induction was observed for **41** (6-Cl) and **42** (6-OCH_3). For **31** (3′-CF_3 on ring B, no ring A substituent) and **46**–**47** (3′-CF_3 on ring B, various ring A substituents), only **47** (6-Cl) improved activity. But in the case of **16** (3′-OH, 4′-OCH_3 on ring B, no ring A substituent) and **43**–**45**

(3′-OH-4′-OCH_3 on ring B, various ring A groups), substitution on ring A did not improve activity. Thus, compounds with 6-Cl on ring A fared better than their unsubstituted ring A analogues only when coupled with certain ring B groups, namely 3′-OCH_3 and 3′-CF_3. This may be true for other substituents as well but with the limited examples available, it is not possible to determine whether ring A or B plays a dominant role.

The activities of the Class 3 compounds were particularly interesting because they shed light on the role of the nitrogen linked Michael acceptor for NQO1 induction. The results showed that it was important to retain an unsubstituted ring nitrogen (*N*-methyl derivatives **49**, **50** were less active) and to keep the carbon–carbon double bond intact. Reduction of the double bond gave compounds without the Michael acceptor moiety (**51**, **52**) and this was accom-

Table 3
CYP1A1 and NQO1 induction ratios of test compounds

Number[a]	Ring A (R)	Ring B (R′)	CYP1A1 induction ratio[b]	NQO1 induction ratio[c]	NQO1/CYP1A1 Quotient[d]
Class 1[e]					
1 (5 μM)	H	H	1.01 ± 0.04	5.13	5.08
2 (1 μM)	H	2′-OCH_3	2.48 ± 0.09	4.03	1.63
3 (1 μM)	H	3′-OCH_3	1.26 ± 0.08	4.21	3.34
4 (5 μM)	H	4′-OCH_3	1.04 ± 0.01	4.50	4.3
5	H	2′,4′ -$(OCH_3)_2$	Not done	—	—
6 (0.05 μM)	H	2′,5′-$(OCH_3)_2$	3.08 ± 0.31	3.32	1.08
7	H	2′,6′-$(OCH_3)_2$	Not done	—	—
8	H	3′,5′-$(OCH_3)_2$	Not done	—	—
9	H	3′,4′,5′-$(OCH_3)_3$	Not done	—	—
10 (7.5 μM)	H	3′ -OC_6H_5	1.08 ± 0.02	3.34	3.09
11 (20 μM)	H	2′-OH	1.02 ± 0.02	3.35	3.28
12 (5 μM)	H	3′-OH	1.11 ± 0.03	3.94	3.55
13 (10 μM)	H	2′,5′-$(OH)_2$	2.87 ± 0.25	7.69	2.68
14 (20 μM)	H	3′,5′-$(OH)_2$	1.04 ± 0.03	3.82	3.67
15 (2.5 μM)	H	2′-OH, 4′-OCH_3	2.35 ± 0.23	3.59	1.53
16 (0.5 μM)	H	3′-OH, 4′-OCH_3	2.66 ± 0.16	3.13	1.17
17 (5 μM)	H	3′-OCH_3, 4′-OH	1.32 ± 0.10	4.00	3.03
18 (5 μM)	H	2′CH_3	1.66 ± 0.10	4.45	2.68
19 (1 μM)	H	3′-CH_3	1.08 ± 0.05	4.04	3.74
20 (7.5 μM)	H	4′-CH_3	1.07 ± 0.04	3.05	2.85
21 (7.5 μM)	H	4′ -C_2H_5	1.08 ± 0.01	4.96	4.59
22 (5 μM)	H	2′-F	1.26 ± 0.10	6.80	5.39
23 (7.5 μM)	H	3′-F	1.11 ± 0.02	4.19	3.77
24 (20 μM)	H	4′-F	1.64 ± 0.09	5.69	3.47
25 (15 μM)	H	2′,4′-$(F)_2$	1.89 ± 0.18	5.06	2.68
26 (0.5 μM)	H	3′-Cl	1.21 ± 0.01	4.47	3.69
27 (2.5 μM)	H	4′-Cl	1.06 ± 0.03	3.67	3.46
28 (10 μM)	H	2′,4′-$(Cl)_2$	1.21 ± 0.06	4.24	3.50
29 (2.5 μM)	H	2′-Cl, 4′-F	1.20 ± 0.04	3.52	2.93
30 (20 μM)	H	2′-CF_3	1.20 ± 0.02	2.96	2.47
31 (0.1 μM)	H	3′-CF_3	1.40 ± 0.09	3.85	2.75
32 (0.1 μM)	H	4′-CF_3	1.22 ± 0.03	2.74	2.24
33 (25 μM)	H	4′-CN	1.03 ± 0.04	2.20	2.13
34 (10 μM)	H	Ring B = 2′pyridinyl	1.08 ± 0.04	3.92	3.63
35 (20 μM)	H	Ring B = 4′pyridinyl	1.01 ± 0.01	2.08	2.06
36 (1 μM)	H	Ring B = 3′indolyl	1.28 ± 0.07	4.33	3.38
37	H	Ring B = 1′ napthalenyl	Not done	—	—
38 (2.5 μM)	H	Ring B = 2′ napthalenyl	1.06 ± 0.01	4.61	4.35
Class 2[e]					
39 (1 μM)	6-F	3′-OCH_3	1.25 ± 0.06	3.21	2.57
40 (2.5 μM)	5-Cl	3′-OCH_3	1.32 ± 0.08	4.34	3.29
41 (0.1 μM)	6-Cl	3′-OCH_3	1.51 ± 0.13	2.56	1.70
42 (0.5 μM)	6-OCH_3	3′-OCH_3	1.13 ± 0.01	3.08	2.72
43 (0.5 μM)	6-F	3′-OH, 4′-OCH_3	2.05 ± 0.07	4.10	2.00
44 (1 μM)	6-Cl	3′-OH, 4′-OCH_3	2.33 ± 0.13	5.09	2.18
45 (1 μM)	6-OCH_3	3′-OH, 4′-OCH_3	1.05 ± 0.01	4.34	4.13
46 (0.05 μM)	6-F	3′-CF_3	1.23 ± 0.08	3.31	2.69
47 (0.05 μM)	6-Cl	3′-CF_3	2.40 ± 0.27	5.20	2.17
48 (0.5 μM)	6-OCH_3	3′-CF_3	1.36 ± 0.07	4.22	3.10
Class 3[f,g,h]					
49[f] (5 μM)	—	3′OCH_3	1.18 ± 0.05	4.78	4.05
50[f] (5 μM)	—	3′-OH, 4′-OCH_3	1.08 ± 0.03	2.64	2.44
51[g] (20 μM)	—	3′-OCH_3	1.78 ± 0.10	2.23	1.25
52[g]	—	4′-CH_3	Not done	—	—
53[h] (5 μM)	—	3′-OCH_3	1.13 ± 0.07	3.88	3.43
54[h] (7.5 μM)	—	3′-OH, 4′-OCH_3	1.38 ± 0.11	4.21	3.05
Class 4[i]					
55 (0.1 μM)	H	H	1.44 ± 0.11	2.80	1.94
56 (5 nM)	6-F	H	2.75 ± 0.14	2.56	0.93
57 (5 nM)	5-Cl	H	2.31 ± 0.15	2.42	1.05
58 (50 nM)	6-Cl	H	2.09 ± 0.06	2.88	1.38
59 (5 nM)	6-CF_3	H	3.01 ± 0.26	2.99	0.99
60 (5 nM)	6-CF_3	5-F	3.72 ± 0.20	3.09	0.83

(*continued on next page*)

Table 3 (*continued*)

Number[a]	Ring A (R)	Ring B (R′)	CYP1A1 induction ratio[b]	NQO1 induction ratio[c]	NQO1/CYP1A1 Quotient[d]
61 (5 nM)	6-CF_3	6-Cl	3.23 ± 0.21	2.96	0.92
Sulforaphane (1 μM)	—	—	0.92 ± 0.04	3.89	4.22
BNF (0.01 μM)	—	—	1.13[j]	1.83	1.62

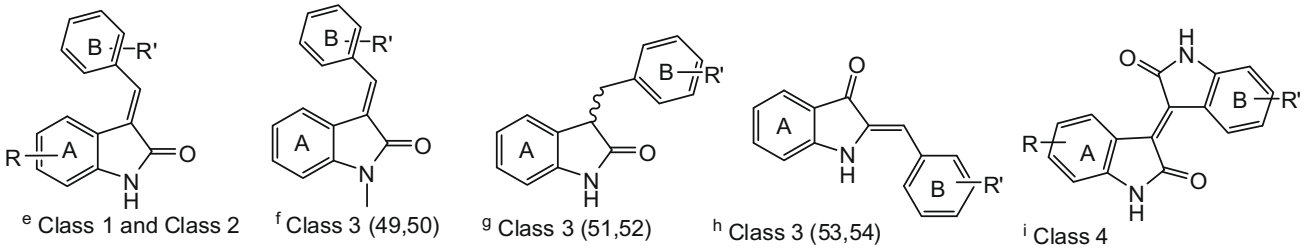
Structures of Class1–4 are as follows:

[a] Compound number and concentration used in CYP1A1 induction assay (≈4 × CD for NQO1 activity) is given in parentheses.

[b] Determined on Hepa1c1c7 cells at concentrations given in column 1. Mean ± SD, $n = 3$. CYP1A1 induction ratio = (Fluorescence$_{\text{Cells+Test Compound}} - F_{\text{Blank}}$)/(Fluorescence$_{\text{Control Cells}} - F_{\text{Blank}}$).

[c] Determined on Hepa1c1c7 cells at the same concentration as CYP1A1 induction ratio. Values were read off from plot of degree of induction versus concentration for each compound.

[d] Quotient = NQO1 induction activity/CYP1A1 induction activity.



[j] This value was read off from a curve constructed with concentrations 0.005, 0.05, 0.5 and 5 μM BNF. Therefore no SD is assigned to this value. At 0.05 μM BNF, CYP1A1 ratio was 1.27 ± 0.12.

panied by a significant drop in NQO1 induction. As mentioned earlier, **53** and **54** differ from **3** and **16** in the positions of the carbonyl and exocylic double bond. In **53** and **54**, the double bond (and not the carbonyl group) is adjacent to the ring nitrogen, while the opposite is true for **3** and **16**. This modification had mixed effects on induction activity, with slightly diminished activities observed for **53** (0.46 μM) and **3** (0.27 μM) but a magnitude difference for **54** (1.9 μM) and **16** (0.12 μM). Thus, of the three modifications investigated in Class 3, reduction of the exocyclic double bond which abolished the Michael acceptor motif had the greatest impact on activity.

The isoindigos of Class 4 were outstanding for their potent induction of NQO1. The increase in size, lipophilicity and rigidity of these compounds compared to the Class 1 and 2 compounds may have contributed to their improved profiles. Isoindigo (**55**) was earlier reported to be a potent NQO1 inducer in Hepa1c1c7 cells (ED_{50} 320 nM).[49] In our hands, it had a CD of 25 nM, but we found greater induction potencies in other members of Class 4 that were substituted with electron withdrawing groups. Subnanomolar CD values were found for **59**, **60** and **61** which had 6-CF_3 on one indole ring and H, 5-F or 6-Cl on the other ring, respectively. They were the most potent NQO1 inducers identified in the entire series.

2.4. Effect of compounds on 7-ethoxyresorufin *O*-deethylase (EROD) activity in Hepa1c1c7 cells

Since induction of NQO1 activity was observed for many Class 1–4 compounds, it was important to determine if phase 1 enzyme activity was induced as well. The EROD assay was used for this purpose.[51] EROD activity describes the rate at which CYP1A1 caused the O-deethylation of 7-ethoxyresorufin to resorufin. If a compound induced CYP1A1 activity, more resorufin would be formed compared to control untreated Hepa1c1c7 cells and the ratio of resorufin fluorescence in treated versus control Hepa1c1c7 cells would increase. This ratio was determined for compounds at concentrations that were approximately 4 times their CD values. A fixed concentration was not used here because it would be difficult to select this concentration in view of the large variation in NQO1 induction activities. The quotient of NQO1 and CYP1A1 induction ratios (ratio determined at the same concentration) was also obtained for each compound. Values >1 would imply greater induction of NQO1 relative to CYP1A1. The results are tabulated in Table 3.

TCDD, BNF and sulforaphane were used as controls in this experiment. TCDD is a known inducer of CYP1A1[49] and in our hands, it gave a ratio of 2.99 in the EROD assay at 1 nM. BNF and sulforaphane are bifunctional and monofunctional NQO1 inducers, respectively.[25] Hence, BNF should induce both CYP1A1 and NQO1 activities in Hepa1c1c7 cells, whereas sulforaphane should induce NQO1 activity to a greater degree than CYP1A1 activity. We found that BNF at 0.01 μM induced NQO1 activity by 1.83 times (NQO1 induction ratio) and CYP1A1 activity by 1.13 times (CYP1A1 induction ratio), thus giving an NQO1/CYP1A1 quotient of 1.62. Others have shown that BNF induced CYP1A1 activity to a greater extent than NQO1 activity but these experiments were based on analyzing the microsomal liver extracts or mRNA levels of these proteins in rats treated with BNF.[52,53] These may have been more sensitive assays than the cell-based determinations used here. On the other hand, the results obtained with sulforaphane were in keeping with its monofunctional character. At 1 μM, it barely induced CYP1A1 activity (ratio = 0.92) but increased NQO1 activity by nearly fourfold (ratio = 3.89).

In general, the Class 1 and 2 compounds were weak inducers of CYP1A1, with only eight compounds (**2**, **6**, **13**, **15**, **16**, **43**, **44**, **47**) increasing CYP1A1 activity by more than twofold. The Class 3 compounds showed that N-methylation (**49**, **50**) had negligible effect but the reduction of the exocyclic double bond (**51**) caused an almost twofold increase in CYP1A1 induction. The 2-benzylideneindolin-3-ones (**53**, **54**) had mixed effects compared to their isomers **3** and **16**, respectively. The isoindigos (**56**–**61**) in Class 4 were strong inducers of CYP1A1, except for the isoindigo **55** which had moderate CYP1A1 induction (1.44). The disubstituted analogues (**60**, **61**) were particularly potent inducers.

More important than the CYP1A1 induction ratio was the quotient of NQO1 versus CYP1A1 induction ratios as this value gave an indication of whether the compound preferentially induced NQO1 to CYP1A1. Strong induction of CYP1A1 may not be unfavourable if it is counter-balanced by even stronger induction of NQO1. Here, we observed an interesting trend when the quotients were compared to the NQO1 induction activity, namely that compounds with larger quotients were actually weaker NQO1 inducers. For example, it was noted that the isoindigo **55** was the least potent NQO1 inducer among the Class 4 compounds (CD 25 nM)

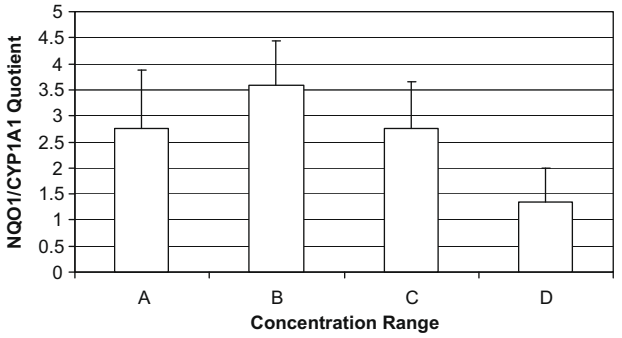


Figure 3. NQO1/CYP1A1 quotients for compounds tested at the following concentration range: (A) 20 and 15 μM; (B) 5–10 μM; (C) 0.1–2.5 μM; (D) 5 and 50 nM. Quotients at all concentration ranges were significantly different ($p < 0.05$, one-way ANOVA, Tukey post-hoc) except those at A and C.

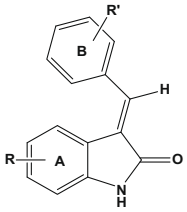
but it had the largest NQO1/CYP1A1 quotient (1.94) in its Class. In Class 1 and 2, the most potent NQO1 inducer was **47** (NQO1 induction ratio 5.20, Table 3) and it had a modest NQO1/CYP1A1 quotient of 2.17. Similar correlations were noted for **6**, **21** and **45**.

To quantify this trend, the compounds were categorized according to the concentrations used to determine the CYP1A1 and NQO1 induction activities. Since the compounds were used at ≈4 × CD for NQO1 activity, weak NQO1 inducers were tested at higher concentrations while strong NQO1 inducers were tested at lower concentrations. In all, 10 concentrations ranging from 20 μM to 5 nM were used and these were grouped into four concentration ranges. Weak inducers (CD 15 and 20 μM, $n = 8$) were found in A and more potent inducers were found in B (CD = 5–10 μM, $n = 17$) and C (CD = 0.1–2.5 μM, $n = 21$). The strongest inducers were grouped in D (CD = 5 and 50 nM, $n = 9$). The mean NQO1/CYP1A1 quotients for each group were compared to other groups by one-way ANOVA with Tukey as post hoc test. Figure 3 shows the quotients for each concentration grouping. Only the quotients obtained at concentration ranges A (15 and 20 μM) and C (0.1–2.5 μM) did not differ significantly from each other at $p < 0.05$. The other comparisons (A vs B or D; B vs C or D; C vs D) showed significant differences. Thus compounds tested at the 5–10 μM range (B) had significantly larger quotients than compounds tested at lower concentration ranges (C and D). The compounds in B were moderately potent NQO1 inducers but preferentially induced NQO1 to CYP1A1. In contrast, the more potent inducers found in category D induced both NQO1 and CYP1A1 to nearly the same extent.

2.5. Antiproliferative activity of compounds on HCT116, MCF7 and CCL186 cells

Class 1–4 compounds were initially screened at a fixed concentration of 10 μM on the human cancer cell lines HCT116 and MCF7 cells. Only eleven compounds (**5**–**7**, **9**, **10**, **37**, **42**, **45**–**48**) caused cell death by more than 50% at this concentration and these compounds were further evaluated for their IC_{50} on the two cancer cell lines (Table 4). The small number (11, 18%) of compounds found to affect cell viability may imply that more stringent requirements exist for antiproliferative activity, as compared to chemoprevention where 85% of members induced NQO1 activity with CD values <10 μM (Table 2).

Table 4
IC_{50} and selectivity ratios of 3-substituted indoline-2-ones on human breast cancer (MCF-7), human colon cancer (HCT116) and normal human diploid embryonic lung fibroblast (CCL-186) cell lines



Compound	Ring A (R)	Ring B (R′)	IC_{50}[a] (μM)		
			MCF-7	HCT-116	CCL-186
5	H	2′,4′$(OCH_3)_2$	8.8 ± 0.5	3.3 ± 0.5	5.8 ± 0.8
			0.7	***1.8***	
6	H	2′,5′$(OCH_3)_2$	5.1 ± 0.1	7.7 ± 0.2	18.1 ± 1.1
			3.5	***2.4***	
7	H	2′,6′$(OCH_3)_2$	4.8 ± 0.7	6.2 ± 0.3	17.8 ± 0.9
			3.7	***2.9***	
9	H	3′,4′,5′- $(OCH_3)_3$	5.8 ± 0.9	10.5 ± 0.7	23.6 ± 1.5
			4.1	***2.2***	
10	H	3′-OC_6H_5	3.7 ± 0.8	8.2 ± 0.7	12.7 ± 0.6
			3.4	***1.5***	
37	H	Ring B = 1′ napthalenyl	3.9 ± 0.6	19.6 ± 0.9	25.0 ± 1.8
			6.4	***1.3***	
42	6-OCH_3	3′OCH_3	1.2 ± 0.4	1.3 ± 0.4	1.8 ± 0.2
			1.5	***1.4***	
45	6-OCH_3	3′OH-4′-OCH_3	3.7 ± 0.3	7.8 ± 0.9	11.3 ± 0.4
			3.1	***1.4***	
46	6-F	3′-CF_3	8.4 ± 0.3	8.9 ± 0.3	7.5 ± 0.7
			0.9	***0.8***	
47	6-Cl	3′-CF_3	3.8 ± 0.3	7.5 ± 0.4	6.6 ± 0.5
			1.7	***0.9***	
48	6-OCH_3	3′-CF_3	1.9 ± 0.5	3.8 ± 0.6	5.4 ± 0.3
			2.8	***1.4***	

[a] Concentration required to reduce cell survival by 50% after 72 h incubation with test compound. Mean ± SD, $n = 3$. Values in bold and italics represent the selectivity ratio: $IC_{50\ CCL186}/IC_{50\ MCF7}$ or $IC_{50\ CCL186}/IC_{50\ HCT116}$.

Only Class 1 and 2 compounds are represented in Table 4. Class 3 compounds which had modifications made to the nitrogen-linked Michael acceptor moiety had negligible antiproliferative activity. This was also true for the isoindigos of Class 4, in spite of their strong induction of NQO1 activity.

The compounds in Table 4 had comparable antiproliferative activities on both cancer cell lines, with IC_{50} values ranging from 1.2 to 19.6 μM. When these activities were compared to those obtained on a normal cell line (human lung fibroblasts), modest selectivities (approximately twofold on average) were observed. Some compounds like **5**, **46** and **47** discriminated poorly between cancerous and normal cells and had selectivity ratios that were close to 1.

For compounds in Class 1, the presence of two or more methoxy groups on ring B strongly favoured activity. Compounds **5**, **6** and **7** are regioisomers with dimethoxy groups on ring B. Their good activity stands in contrast to another regioisomer **8** (3′,5′-OCH_3 on ring B) which had negligible activity and was not short-listed for IC_{50} determination. This may mean that there are preferred positions for dimethoxy groups on ring B. The other Class 1 compounds (**9**, **10**, **37**) in Table 4 have in common bulky substituents on ring B or a bulky ring B. Compound **9** has three methoxy groups on ring B (3′,4′,5′-OCH_3). A 4′-phenoxy group is present in **10**. In **37**, ring B was replaced by 1′-napthalenyl. Interestingly the 2′-napthalenyl isomer **38** was not found to have significant antiproliferative activity. **37** and **38** were also observed to have very different NQO1 induction activities, but with **38** (CD 0.42 μM) more potent than **37** (CD >19 μM). These examples underscore the different structural requirements among Class 1 compounds for these two activities.

The Class 2 compounds are strongly represented for antiproliferative activity. Of the 10 Class 2 compounds available, half of them were found to have antiproliferative activity. As stated earlier, the Class 2 compounds had only three different ring B substituents (3′-OCH_3, 3′-OH-4′- OCH_3, 3′-CF_3). Interestingly, all members with 3′-CF_3 (**46**, **47**, **48**) were listed in Table 4, indicating the important role played by this substituent, quite independently of the ring A substituent. Similarly, ring A of Class 2 had only 3 different substituents at position 6, namely 6-OCH_3, 6-Cl and 6-F. Of these, 6-OCH_3 appears to be preferred because all three compounds (**42**, **45**, **48**) with 6-OCH_3 are listed in Table 4, irrespective of the group on ring B. Hence, it may be concluded that 6-OCH_3 (ring A) and 3′-CF_3 (ring B) are preferred groups for antiproliferative activity, notwithstanding that the most active compound **42** (6-OCH_3, 3′-OCH_3) did not have both features in the same molecule. The substituent preferences for antiproliferative activity are comparatively well defined, unlike NQO1 induction activity as described in Section 2.3.

2.6. Effect of selected Class 1 and 2 compounds on cell cycle of HCT116 cells

To have a better understanding of the mechanisms underlying the antiproliferative activities of the compounds listed in Table 4, their effects on the cell cycle was investigated by fluorescence-activated cell sorter (FACS) analysis using flow cytometry. The experiments were designed to examine the effects of the test compounds on synchronized cell populations at the G1 or G2/M phase. G1 synchronized cells were obtained by growing cells to confluence for up to 5 days and then stimulating their re-entry into the cell cycle by sub-culturing at lower densities. After 24 h of growth, these cells would be aligned at the G2/M phase.

Cells released from the G1-block were exposed to a fixed concentration (≈1.5 × IC_{50} on HCT116 cells) of test compound for 24 h, after which the distribution of cells at the different phases were determined, and compared to control untreated cells. Cells aligned at the G2/M phase were similarly treated. Representative FACS diagrams of control cells released from G1 block and G2 block are give in Figure 4. The proportion of cells in each phase after release from G1- or G2-arrest are given in Table 5.

Analysis of the results revealed that the compounds caused either G1 arrest (**9**, **10**, **46**, **47**) or G2 arrest (**5**–**7**, **42**, **45**, **48**). The exception was **37** which did not interfere with cell cycle progression. Its antiproliferative activity may arise from other mechanisms like autophagy or necrosis. Taking **46** as an example of a compound associated with G1 arrest, the FACS diagram showed that when cells released from G1 block were treated with **46**, the progression

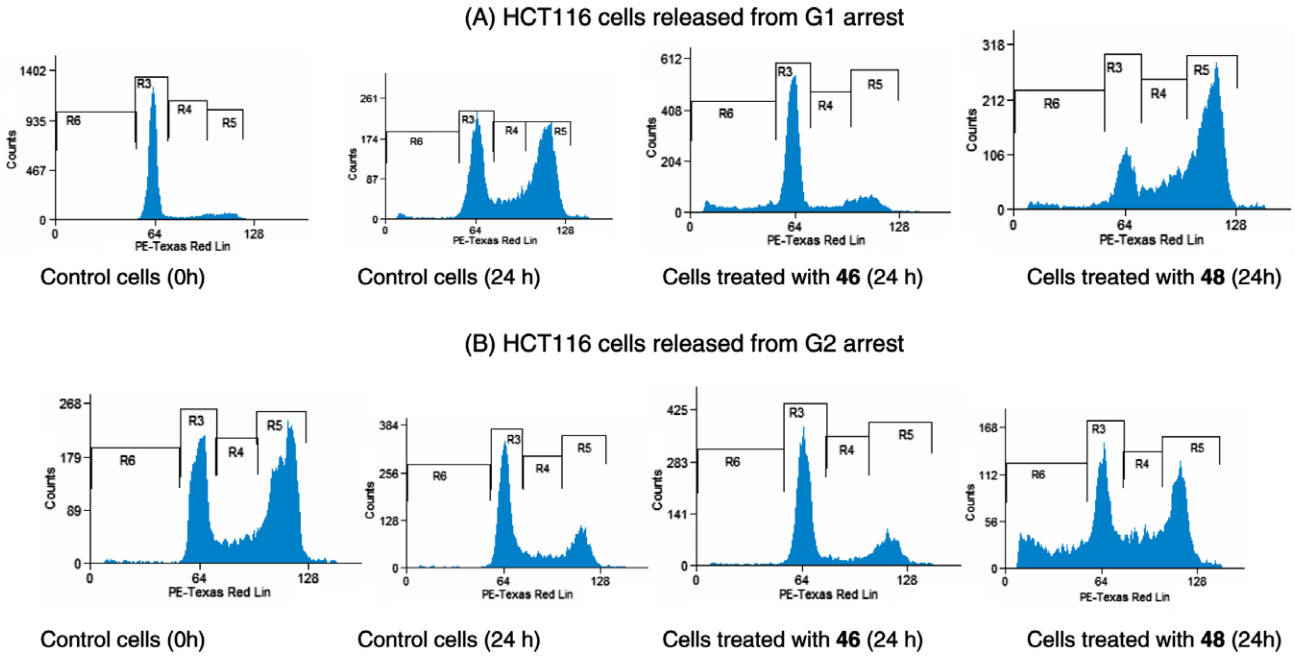


Figure 4. FACS diagrams showing effects of representative compounds **46**, **48** on HCT116 cells released from (A) G1-block and (B) G2-block. R6, R3, R4, R5 refer to sub-G1, G1, S and G2/M phases, respectively. **46** and **48** were tested at 15 μM and 5 μM, respectively (≈1.5 × IC_{50}).

Table 5
Effect of test compounds on various phases of cell cycle

Compound[a]	Sub-G1	G1	S	G2/M
HCT 116 cells released from G1 block[b]				
Control (0 h)	0.46	80.19	6.8	11.57
Control (24 h)	1.94	34.03	14.90	49.47
5 (5 μM)	2.64	26.14	12.37	58.82
6 (10 μM)	2.64	24.24	26.68	46.51
7 (10 μM)	3.52	20.86	16.00	59.63
9 (15 μM)	3.78	56.85	21.42	18.54
10 (15 μM)	3.62	55.93	21.83	18.98
37 (30 μM)	2.96	32.69	26.91	38.42
42 (2.5 μM)	4.22	10.56	13.81	71.54
45 (15 μM)	5.26	17.49	21.13	56.49
46 (15 μM)	12.68	64.93	8.04	14.58
47 (15 μM)	12.39	65.55	7.10	15.13
48 (5 μM)	4.39	17.50	19.12	59.16
HCT 116 cells released from G2 block[b]				
Control (0 h)	1.17	31.12	12.11	55.97
Control (24 h)	1.24	55.88	14.93	27.90
5 (5 μM)	12.49	34.66	13.43	39.43
6 (10 μM)	9.83	39.33	16.68	34.26
7 (10 μM)	9.51	32.97	12.42	44.99
9 (15 μM)	4.13	42.06	16.84	36.45
10 (15 μM)	2.16	63.51	13.10	21.09
37 (30 μM)	5.68	51.32	14.61	28.26
42 (2.5 μM)	30.54	26.01	15.36	43.57
45 (15 μM)	15.54	26.01	15.36	43.57
46 (15 μM)	2.49	64.15	8.82	25.00
47 (15 μM)	10.62	53.47	8.05	28.39
48 (5 μM)	20.69	30.55	17.96	31.58

[a] Concentration used is indicated in brackets. $\approx 1.5 \times IC_{50\ HCT116}$.
[b] Proportion of cells in each phase was the mean of 3 separate determinations.

from G1 to G2/M was interrupted (Fig. 4A). Notable increases in the sub-G1 phase were observed (Table 5). As anticipated, **46** had no effect on the transition of cells from G2/M to G1 (Fig. 4B). In the case of **48** which caused G2-arrest, it did not interfere with the progression of cells released from G1 (Fig. 4A) but prevented cell transit from G2/M to G1 (Fig. 4B). Thus, the proportion of cells in G1 did not increase after exposure (24 h) to **48** (30.55% in G1, compared to 55.88% in untreated cells) (Table 5). There were also significant increases in apoptotic cells in the sub-G1 phase (Fig. 4B, Table 5).

A structural trend was observed among the compounds that caused G1 or G2 arrest. Compounds (**5**–**7**, **42**, **45**, **48**) that caused G2 arrest had in common the presence of one or two methoxy groups on ring A or B**.** Compounds **5**–**7** are regioisomers with two methoxy groups on ring B. **42**, **45** and **48** had 6-OCH_3 on ring A. Rings B of **42** and **45** were also methoxylated. By contrast, compounds (**9**, **10**, **46**, **47**) that caused G1 arrest were either halogentated (**46**, **47)** or had bulky ring B substitutents (**9**, **10**). The outlier status of **37** which caused neither G1 nor G2 arrest is not unexpected since it is structurally different from the other compounds.

3. Discussion

Our results show that functionalized 3-benzylideneindolin-2-ones were inducers of NQO1, a Phase II enzyme that is widely used as a biomarker for cancer chemoprevention. The Class 1 and 2 compounds ($n = 48$) yielded an impressive 5000-fold variation in induction activity, with several members reporting CD values in the nanomolar range. An analysis of structure–activity relationships highlighted the critical role of the nitrogen-linked Michael acceptor motif for activity. The reduction of the exocyclic double bond effectively abolished the Michael acceptor feature and resulted in a significant lowering of activity whereas modifications that left this feature intact, like N-methylation and switching the positions of the carbonyl and double bond, decreased activity to a lesser degree. The isoindigos in Class 4 were by far the most potent NQO1 inducers in this study. It is tempting to attribute their strong activities to the presence of two Michael reaction moieties embedded in the isoindigo template but this would require verification. The unique physicochemical profiles of isoindigos in terms of size, lipophilicity and conformational rigidity may play a role as well.

Having established a structural role for the Michael acceptor moiety, we proceeded to investigate its functional contribution to NQO1 induction. Compounds with Michael acceptor moieties are generally associated with the induction of NQO1 without concurrent induction of CYP1A1 activity (mono-functional inducers). Our results showed that the compounds also induced CYP1A1 activity in the Hepa1c1c7 cells, albeit to varying degrees. Class 4 isoindigos were particularly strong inducers of CYP1A1 (quotients $\leqslant 1$) and there were others (**2**, **6**, **15**, **16**, **41**, **51**) that induced CYP1A1 and NQO1 to almost the same extent (quotients < 2). It is tempting to attribute this profile to the presence of the nitrogen in the Michael acceptor moiety because indoles like indole-3-carbinol and brassinin are known to be bifunctional inducers and should induce both NQO1 and CYP1A1.[2] Nonetheless, it was heartening to note that there were as many compounds (**1**, **4**, **21**, **38**, **45**, **49**) that induced NQO1 to a greater extent than CYP1A1 and with quotients that were comparable or exceeded that of sulforaphane (1 μM, quotient = 4.22), a known mono-functional NQO1 inducer. Analysis of the NQO1/CYP1A1 quotients also revealed an interesting trend in that compounds with favourable quotients (>3) were generally those with moderate NQO1 induction activity (CD ≈ 1–2 μM). In contrast, compounds that were very strong or weak NQO1 inducers tend to discriminate poorly between the two proteins.

Recent investigations revealed evidence of cross-talk between the AhR/XRE (associated with bi-functional induction) and Keap1/Nrf2/ARE (associated with mono-functional induction) signaling pathways and the coordinate regulation of the AhR and Nrf gene batteries.[54–56] The expression of NQO1 would then be regulated by these interactions.[6,31] If so, the distinction between mono- and bi-functional inducers may not be as clear-cut and the perceived advantages of mono-functional over bi-functional inducers may be oversimplified.

Turning to the antiproliferative activities of the Class 1–4 compounds, only a small number of compounds were found to cause at least 50% cell death at 10 μM. It would seem that the structural templates of Class 1–4 are better suited for chemoprevention than antiproliferative activity. Nonetheless, the presence of an intact nitrogen-linked Michael acceptor moiety remains a requirement for both activities. Within each class, we noted differences in structural requirements. For example, Class 1 compounds with alkoxy groups (dimethoxy, trimethoxy) groups on ring B had good antiproliferative activities but were not particularly outstanding for NQO1 induction. Among Class 2 compounds, 3′-CF_3 on ring B and 6-OCH_3 on ring A were important substitutents for antiproliferative activity but preferences for NQO1 induction were less clear cut. However, we did identify a small number of compounds that combined good antiproliferative activity with chemopreventive potential (as evaluated from their NQO1/CYP1A1 quotient). These were mostly Class 2 compounds (**42**, **45**–**48**) and one compound from Class 1 (**10**). These compounds had NQO1/CYP1A1 quotients that exceeded 2. The Class 2 compounds (**42**, **45**–**48**) are particularly promising because they have CD values in the nanomolar range but yet show at least twofold selectivity for NQO1 induction. These compounds could potentially have the dual effects of cytoprotection towards normal cells while being cytotoxic to cancer cells.

An unexpected finding was how structural features in the compounds with antiproliferative activity were linked to their effects on cell cycle progression. Compounds with at least two methoxy groups on rings A and B (**5**–**7**, **42**, **45**) were found to cause G2 arrest, possibly by interfering with the formation of the mitotic spindle. By contrast, compounds with bulky substituents (trimethoxy, phenoxy) on ring B (**9**, **10**) and halogenated groups on both rings (**46**–**48**) caused G1 arrest which may involve inhibition of cyclin dependent kinase activity. Evidence of apoptosis was observed for these compounds. Compound **37**, a structural outlier, did not disrupt the cell cycle at either phase. These structural trends serve to highlight how seemingly small variations on the ring structure could affect biological responses.

In conclusion, we have demonstrated the chemopreventive potential of benzylideneindolinones as seen from their ability to induce NQO1 activity. 85% of the compounds evaluated had CD values that were less than 10 μM, highlighting the potential of this scaffold for chemoprevention. As anticipated, SAR pointed to the importance of an intact nitrogen linked Michael acceptor moiety for this activity. On the other hand, the presence of the nitrogen may have predisposed the compounds to the induction of both CYP1A1 and NQO1 activities although there were candidates that selectively induced NQO1 by fourfold or more in this series. In the case of the antiproliferative activity, only a handful of compounds were able to successfully combine selective induction of NQO1 with good antiproliferative activity ($IC_{50} < 10$ μM on two cancer cell lines). Most of these compounds were from Class 2 and even among this small number (**42**, **45**–**48**), interesting differences in the way they affected the cell cycle were noted. They are potentially useful leads for compounds that combine antiproliferative activity against cancer cells with a degree of cytoprotection mediated through NQO1 induction in normal cells.

4. Experimental

4.1. Chemistry

Melting points (uncorrected) were determined on a melting point apparatus in open glass capillary tubes. ^{1}H and ^{13}C NMR spectra were measured on Brucker ACF (DPX-300) (^{1}H at 300 MHz, ^{13}C at 75 MHz) magnetic resonance spectrometer. ^{1}H chemical shifts were reported in ppm using residual $CHCl_3$ (δ 7.25) and DMSO (δ 2.49) as internal standards. Coupling constants (J) were reported in Hertz (Hz). Proton decoupled ^{13}C NMR spectra were reported in ppm (δ) relative to residual $CHCl_3$ (δ 77.0) and DMSO (39.5). Reactions were routinely monitored by thin layer chromatography (TLC) on pre-coated plates (Silica Gel 60 F254, Merck), with ultraviolet light as visualizing agent. Column chromatography was carried out with Silica Gel 60 (0.04–0.063 mm) with hexane/ethyl acetate as eluting solvents. Nominal mass spectra were collected on LcQ Finnigan MAT mass spectrometer with chemical ionization (APCI) as probe. The purity of final compounds was verified by reverse phase HPLC on two different solvent systems (isocratic mode) and details are given in Supplementary data, which also contains information on the melting points, nominal mass and NMR data of the final compounds (**1**–**61**).

4.2. General procedure for synthesis of 3-substituted indolin-2-ones (1–48)

Equimolar amounts (0.5 mmol) of the oxindole and aldehyde were dissolved in ethanol (10 ml), a drop of piperidine (20 μl) was added and the mixture heated in a sealed vessel (10 ml) flushed with argon. The vessel was heated to 140 °C for 15 min in a microwave synthesizer (Biotage Initator®). Compounds **1**–**5**, **7**–**9**, **14**, **18**, **22**, **20**, **24**, **27**, **33**–**36** were synthesized by conventional heating (reflux, 18 h) using the same quantities of reactants dissolved in a larger volume (20 ml) of ethanol. The reaction mixture was then cooled to room temperature and the resulting precipitate removed by filtration, carefully washed with cold ethanol and recrystallized at least once from ethanol to give the desired product. Some compounds (**2**, **5**, **6**, **9**, **12**, **13**, **22**) were not sufficiently soluble in ethanol to permit recrystallization. These compounds were washed many times with ethanol until TLC showed a single spot. In the case of **14**, it did not precipitate from the reaction mixture on cooling. The solvent was then removed under vacuum and the oil purified by column chromatography (hexane/ethyl acetate = 4:1) until TLC gave a single spot.

4.3. General procedure for synthesis of substituted 3-benzylindolin-2-ones (51, 52)

Compound **20** or **41** (100 mg) was dissolved in ethanol (15 ml), 10 mg Pd/C (10%) was added and the mixture was reacted with H_2 in a shaking Parr Hydrogenator at 50 psi, 16 h, room temperature (30 °C). The catalyst was removed by filtration. The solvent was removed under reduced pressure on a rotary evaporator to give the crude product which was purified by column chromatography (hexane/ethyl acetate = 3:1) or recrystallization from ethanol.

4.4. General procedure for synthesis of substituted 2-benzylidene indolin-3-ones 53 and 54[57]

1-(3-Hydroxy-1*H*-indol-1-yl)ethanone (5 mmol) was dissolved in 50% aqueous ethanol (25 ml) containing concentrated HCl (0.5 ml) and heated to reflux for 1 h under protection of argon. The aldehyde (5 mmol) in ethanol was then added and heating was continued for another 2 h. The reaction mixture was cooled to room temperature, after which the solvent was removed under reduced pressure and the residue purified by column chromatography (hexane/ethyl acetate = 4:1).

4.5. General procedure for synthesis of isoindigos 55–61[58]

A solution of the substituted indolin-2-one (oxindole) (0.5 mmol) and indoline-2, 3-dione (isatin, 0.5 mmol) in glacial acetic acid (20 ml) and concentrated hydrochloric acid (0.5 ml) was heated to reflux for 16 h under argon protection. The reaction mixture was cooled to room temperature and the precipitate removed by vacuum filtration and washed liberally with ethyl acetate until a single spot was detected on TLC.

4.6. X-ray crystallography

Crystals of **21** or **37** were grown in ethanol and mounted on glass fibres. X-ray data were collected with a Bruker AXS SMART APEX diffractometer, using Mo Kα radiation at 223 K, with the SMART suite of Programs (SMART version 5.628 (200), Bruker AXS Inc., Madison, WI). Data was processed and corrected for Lorentz and polarisation effects with SAINT (SAINT+ version 6.22a (2001) Bruker AXS Inc., Madison, WI), and for absorption effect with SADABS (SADABS, version 2.10, 2001, University of Göttingen). Structural solution and refinement were carried out with the SHELXTL, suite of programs (SHELXTL, Version 6.14 (2000), Bruker AXS Inc., Madison, WI). The structure was solved by direct methods to locate the heavy atoms, followed by difference maps for the light, non-hydrogen atoms. All non-hydrogen atoms were generally given anisotropic displacement parameters in the final model whereas H-atoms were placed at calculated positions. X-ray crystal data of **21** and **37** are given in Supplementary data.

4.7. Materials for biological assays

Menadione, digitonin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), β-naphthoflavone (BNF), dicuomarol, salicylamide, propidium iodide, RNase A, 7-ethoxyresorufin and sulforaphane were purchased from Sigma–Aldrich (St. Louis, MO). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was purchased from AccuStandard (New Haven, USA). Other reagents were of analytical grade.

4.8. Determination of NQO1 activity[14]

Hepa1c1c7 were purchased from American Type Culture Collection (Rockville, MD). and cultured in alpha-minimum essential medium (α-MEM) without nucleosides, and containing 10% (v/v) heat- and charcoal-treated fetal calf serum (1 g of charcoal per 100 ml of serum; 90 min at 5 °C), 0.15% sodium bicarbonate, 0.01% penicillin G, 0.01% streptomycin sulfate in an humidified atmosphere of 5% CO_2 at 37 °C. Cells were sub cultured when they reached 80–90% confluency and used within 6–17 passages for determinations. For the assay, about 10,000 cells were grown in each well of a 96-well plate for 24 h in α-MEM. Total protein content per was found to be 0.036 mg per well based on the Bradford assay. Stock solutions of test compounds were prepared in DMSO and aliquots were added to each well to give the desired concentration. The final concentration of DMSO in each well was kept at 0.5% v/v or lower. After incubation for 48 h, the media was decanted and the cells lyzed by a solution containing 0.8% w/v digitonin and 2 mM EDTA with incubation at 37 °C (10 min). The plates were gently agitated on an orbital shaker for 10 min at 23 °C. An aliquot (200 μl) of a solution ('complete reaction mixture') was then added to each well. This solution was freshly prepared just prior to use and consisted of 7.5 ml 0.5 M Tris–Cl (pH = 7.4), 100 mg bovine serum, 1 ml 1.5% Tween-20, 0.1 ml 7.5 mM FAD, 1 ml 150 mM glucose 6-phosphate, 90 μl 50 mM NADP, 300 units yeast glucose-6-phosphate dehydrogenase, 45 mg MTT and deionized water made up to a final volume of 150 ml. Menadione (1 μl of 50 mM menadione dissolved in acetonitrile for every milliliter of reaction mixture was added just before the mixture was dispensed into the wells. After addition, the plate was gently agitated for 5 min and the reaction was quenched by addition of a solution (50 μl) of 0.3 mM dicoumarol in 0.5% DMSO and 5 mM potassium phosphate, pH = 7.4. A blue colour due to the formation of formazan was observed in each well, the absorbance of which was measured at 590 nm on a plate reader. Blank wells contained no cells and control wells contained Hepa1c1c 7 cells treated with medium containing 0.5% DMSO but without the test compound. NQO1 induction activity of a compound tested at a given concentration was determined from the equation:

$$\text{Degree of induction} = A_{\text{test compound}} - A_{\text{blank}} / A_{\text{control}} - A_{\text{blank}}$$

where *A* is absorbance of formazan measured at 590 nm.

Sulforaphane and BNF were determined under similar conditions as positive controls. The results were plotted (degree of induction vs concentration) with OriginPro 7.5 SR1 (Version V7.5776 B776), OriginLab Corporation, MA and the concentration of test compound required to double the basal NQO1 activity (CD) was determined. Three separate determinations were made and CD was reported as mean ± SD for $n = 3$.

4.9. Determination of 7-ethoxyresorufin *O*-deethylase (EROD) activity in Hepa1c1c7 cells[59]

Hepa1c1c7 cells were plated at a density of 10,000 cells per well in a 96-well plate and cultured for 24 h. A stock solution of test compound was prepared in DMSO and serially diluted with medium to give the desired concentration in the well. The final concentration of DMSO was 0.5% v/v. The cells were incubated with test compound for 48 h, after which the medium was removed, the well washed with 200 μl of 1×phosphate-buffered saline solution (PBS) and then incubated with 5 μM 7-ethoxyresorufin and 2 mM salicylamide in 200 μl of medium at 37 °C, 40 min. Readings were taken at $\lambda_{\text{excitation}}$ of 530 nm and $\lambda_{\text{emission}}$ of 590 nm on a fluorometer. Fluorescence (if any) of the test compound was determined at the same wavelengths to take into account its contribution to the observed readings. Readings of empty wells (no cells, 'blank') and wells with Hepa1c1c7 cells in medium containing 0.5% DMSO but without test compound ('control') were also determined. TCDD, a strong inducer of CYP1A1 activity, as well as BNF and sulforaphane were used as controls.

CYP1A1 induction activity was given by the expression:

$$\text{Degree of induction} = F_{\text{Cells+test compound}} - F_{\text{Blank}} / F_{\text{Control}} - F_{\text{Blank}}$$

4.10. Determination of antiproliferative activity by the microculture tetrazolium (MTT) assay[60]

The antiproliferative activity of the test compounds were determined by the MTT assay on the following cell lines: MCF7 (human breast cancer cell line), HCT116 (human colon cancer cell line) and CCL186 (normal human diploid embryonic lung fibroblast). The cell lines were purchased from American Type Culture Collection (Rockville, MD). MCF7 and CCL186 cells were cultured in Eagle's Minimum Essential Medium (EMEM) with 1 mM sodium pyruvate, 1.5 g/l sodium bicarbonate, 2 mM L-glutamine supplemented with10% FBS and 0.01% antibiotics. HCT116 cells were cultured in McCoy's 5A Medium with 2.2 g/l sodium bicarbonate supplemented with 10% FBS and 0.01% antibiotics. Cells were plated at the following densities: 10,000 cells/well (MCF7), 4000 cells/well (HCT116), 5000 cells/well (CCL186). They were grown for 24 h in a 96-well plate, with 100 μl of corresponding medium in each well. The test compounds were prepared in DMSO and diluted to a series of concentrations with medium. Not more than 1% DMSO (final concentration) was present in each well. The test compounds were incubated with the cells for 72 h, after which 100 μl MTT solution (0.5 mg/ml in 1 × PBS) was added for 3 h and the cells lysed to release the formazan product. The latter was dissolved in DMSO (150 μl) and absorbance determined within 30 min at 590 nm on a microtitre plate reader. Cell survival was given by the expression:

$$\text{Cell survival } (\%) = [(A_{\text{cells+test compound}} - A_{\text{blank}}) / (A_{\text{untreated cells}} - A_{\text{blank}})] \times 100$$

where A is the absorbance of formazan measured at 590 nm in the test ($A_{\text{cells + test compound}}$), control ($A_{\text{untreated cells}}$) or blank ($A_{\text{blank}}$) wells. Each concentration of test compound was evaluated on 3 separate occasions. The concentration (IC_{50}) that inhibited 50% of cell growth was determined from the sigmoidal curve obtained by plotting % surviving cells versus concentration using OriginPro 7.5 SR1 (Version V7.5776 B776), OriginLab Corporation, MA.

The MTT assay was also carried out on Hepa1c1c7 cells for the purpose of determining cytotoxicity of test compounds. The same procedure was adopted except that the incubation period of compound with cells was 48 h, instead of 72 h.

4.11. Determination of the effects of test compounds on the cell cycle of HCT116 cells by cell cytometry

HCT116 cells were grown to confluence and maintained in this state for at least 5 days without change of media. The serum starved cells were then trypsinized and subcultured at densities

of 5×10^5 cells/well in six-well plates. Growth media contained 10% fetal calf serum. The test compound was added either immediately to cells synchronized at G1 phase, or 24 h later when cells were synchronized at G2 phase. The cells were incubated with test compound for 24 h from the time of addition. After this time, the cells were harvested, trypsinized and fixed in 70% ice-cold ethanol for a minimum of 24 h. After centrifugation, the supernatant was discarded and the pellet was treated with RNase A (200 μg/ml) for 30 min at room temperature, followed by cell staining using propidium iodide at a final concentration of 20 μg/ml. The stained cells were then analyzed for cell cycle distribution in the sub-G1, G1, S and G2/M phases on a Dako Cytomation Cyan LX (Dako Colorado, Fort Collins, CO, USA) equipped with an argon solid state laser (488 nm) using the Summit (version 4.3) software. Compounds were tested at a concentration of $\approx 1.5 \times IC_{50}$, with IC_{50} determined by the MTT assay on HCT116 cells.

4.12. Statistical analysis

Data was analyzed for statistical significance by one-way ANOVA followed by Tukey HSD as post-hoc test on SPSS 15.0 for Windows, Chicago, IL. Spearman's correlation analysis was carried out on the same software. A level of probability of 0.05 was used as the criterion for significance.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.12.052.

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