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Methoxylation of resveratrol: Effects on induction of NAD(P)H Quinone-oxidoreductase 1 (NQO1) activity and growth inhibitory properties

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ABSTRACT

A series of methoxystilbenes (*E* and *Z* isomers) related to resveratrol were investigated for their effects on NQO1 induction in murine hepatoma cells and growth inhibitory effects on human cancer cell lines. Both activities were enhanced in compounds with methoxy groups on rings A and B of resveratrol but methoxylation of the di-meta (3,5) hydroxyl groups on ring A of resveratrol was found to be more critical for improving activity. Strikingly different structure–activity trends were observed, namely the association of *E* isomers with potent NQO1 induction activity and *Z* isomers with growth inhibitory properties. The introduction of *ortho*-methoxy groups on ring A greatly benefited NQO1 induction activity while *meta*/*para* methoxy groups on ring A were preferred for potent growth inhibitory effects. These results serve to highlight the contrasting effects on different activities brought about by methoxylation, which is widely employed as a structural modification approach to improve potency and bioavailability of resveratrol. It serves as a timely reminder that in the course of structural modification, a balance between optimizing desired outcomes against unwanted effects is necessary and the most potent analog need not always be the most desirable.

The phytoalexin resveratrol (3,5,4′-trihydroxystilbene) is reputed to have several beneficial effects on human heath, such as a role in the prevention of heart disease, neurodegeneration and cancer.[1–3] However, resveratrol has several pharmacokinetic limitations, namely its susceptibility to phase II metabolism (glucuronidation and sulfation of the hydroxyl groups) leading to a short half-life and limited bioavailability.[4] Moreover, the pharmacological effects of resveratrol are observed at relatively high concentrations. For example, its growth inhibitory effects were reported at 40–200 μM[5] and it induced the phase II enzyme NAD(P)H quinone-oxidoreductase 1 (NQO1), commonly used as a biomarker for identifying compounds with chemopreventive potential[6], at approximately 20 μM. We have previously reported the synthesis of *E* and *Z* methoxy analogs of resveratrol and investigated their potential as chemopreventive agents based on the induction of NQO1 activity in murine hepatoma cells (Hepa1c1c7).[7] We found that several methoxy analogues, notably those with *ortho*-methoxy groups, were more potent inducers of NQO1 than resveratrol. However, as the induction of NQO1 was determined at different concentrations of test compounds, we were not able to provide a reliable and quantitative comparison with resveratrol. In this report, NQO1 activities of the test compounds were quantified in terms of the concentration required to increase NQO1 activity by twofold (CD) compared to basal levels of activity observed in untreated Hepa1c1c7 cells. We found that the promising induction activities of the *ortho*-methoxy analogs were greater than expected, with the most promising compound **3E** being at least 20-fold more potent than resveratrol as an inducer of NQO1. The unexpected variation in activity arising from seemingly minor changes to structure prompted a more detailed analysis of structure–activity relationships which is described in the following paragraphs.

The CD values of the test compounds are given in Table 1. Resveratrol is a weak inducer of NQO1.[7] Earlier, we noted that it induced NQO1 by a modest 1.2-fold at 12.5 μM. Here, resveratrol failed to induce NQO1 activity by twofold even at 20 μM, which led us to assign a CD of >20 μM to it. Resveratrol has three hydroxyl (OH) groups, with two OH groups at 3 and 5 (*meta*) positions of ring A, and a single OH at the 4′ (*para*) position on ring B. Methoxylation of the OH groups on ring A or B influenced induction activity in different ways. With respect to the 4′-OH group on ring B, it may be removed or methoxylated without significant effect on induction activity. This is seen from the poor activity of pinosylvin in which the 4′-OH group was removed (CD >20 μM) and compounds **11E/Z** to **14E/Z** (CD >20 μM) in which the 4′-OH group was methoxylated. In these compounds, the OH groups on ring A were retained.

Interestingly, when the OH groups on ring A were methoxylated (along with the 4′-OH on ring B), induction activity was significantly improved. This was seen from the markedly improved induction activities of the ring A methoxylated analogs **3E**, **4E**, **6E**

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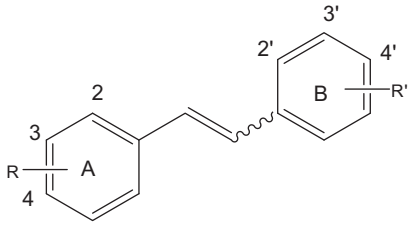
Table 1
IC_{50} and CD values of resveratrol and methoxylated analogues on Hepa1c1c7 cells



Compound	R on Ring A	R′ on Ring B	IC_{50}[a] (μM)	CD[b] (μM)
3E	2-OCH_3	4′-OCH_3	>25	0.85
3Z	2-OCH_3	4′-OCH_3	11.5	No induction at IC_{50}[c]
4E	3-OCH_3	4′-OCH_3	>25	3.1
4Z	3-OCH_3	4′-OCH_3	9.7	No induction at IC_{50}[c]
5E	4-OCH_3	4′-OCH_3	>25	>20[d]
5Z	4-OCH_3	4′-OCH_3	>25	>20[d]
6E	2,4-OCH_3	4′-OCH_3	>25	5.0
6Z	2,4-OCH_3	4′-OCH_3	8.1	No induction at IC_{50}[c]
7E	3,4-OCH_3	4′-OCH_3	>25	>20[d]
7Z	3,4-OCH_3	4′-OCH_3	5.1	No induction at IC_{50}[c]
8E	3,5-OCH_3	4′-OCH_3	5.2	No induction at IC_{50}[c]
8Z	3,5-OCH_3	4′-OCH_3	0.59	No induction at IC_{50}[c]
9E	2,6-OCH_3	4′-OCH_3	10.3	2.0
10E	3,4,5-OCH_3	4′-OCH_3	1.0	No induction at IC_{50}[c]
10Z	3,4,5-OCH_3	4′-OCH_3	0.034	No induction at IC_{50}[c]
11E	2-OH	4′-OCH_3	>25	>20[d]
12E	3-OH	4′-OCH_3	>25	>20[d]
12Z	3-OH	4′-OCH_3	>25	>20[d]
13E	3,5-OH	4′-OCH_3	>25	>20[d]
13Z	3,5-OH	4′-OCH_3	>25	>20[d]
14E	3-OH, 4-OCH_3	4′-OCH_3	>25	>20[d]
14Z	3-OH, 4-OCH_3	4′-OCH_3	>25	>20[d]
15E	2-OCH_3	2′-OCH_3	>25	2.5
Resveratrol	3,5-OH	4′-OH	>25	>20[d]
Pinosylvin	3,5-OH	H	>25	>20[d]
Stilbene (*E*)	H	H	>25	17.7
Stilbene (*Z*)	H	H	>25	>20[d]

[a] Concentration required to reduce cell survival of Hepa1c1c7 cells by 50%. Mean of three determinations.[15] IC_{50} could not be determined for some compounds because of their poor solubilities in the test media.
[b] Concentration required to increase NQO1 induction activity by twofold compared to basal levels observed in untreated Hepa1c1c7 cells. Mean of three determinations. CD of reference compounds sulforane and BNF were 0.15 μM and 0.013 μM, respectively.[13]
[c] No induction observed at concentrations $\leqslant IC_{50}$.
[d] CD was not determined because twofold induction was not observed at 20 μM and solubility problems were encountered if compound was tested at higher concentrations.

and **9E**, which serves to highlight an important structure–activity relationship feature, namely that OH groups on ring A are a liability to induction activity. However, there are caveats to the potency enhancing effects of ring A methoxy groups. These relate to the position of the methoxy groups and the stereochemistry of the double bond.

With regards to the position of the methoxy groups, there appears to be a preference for the ortho position 2 on ring A. Thus, good induction activity was found for **3E** (2-OCH_3, CD 0.85 μM), **6E** (2,4-OCH_3, CD 5.0 μM) and **9E** (2,6-OCH_3, CD 2.0 μM). The exception is **4E** which was reasonably active although it has a methoxy group at the *meta* position (position 3) of ring A. With regards to the stereochemistry of the double bond, there is a noticeable association of strong induction activity with the *E* isomer. Thus, the corresponding *Z* isomers of **3E**, **6E** and **9E** were inactive as inducers at their growth inhibitory concentrations (IC_{50}) on Hepa1c1c7 cells. The preference for the *E* isomer was also noted for the unsubstituted stilbene, where *E*-stilbene had a measurable if modest CD value (17.7 μM) as compared to its *Z* isomer for which CD >20 μM was assigned. Taken together, these observations broadly support a more prominent role for methoxy groups on ring A as a means of enhancing induction activity, as compared to the same groups on ring B. The preference for *ortho*-methoxy on ring A is not readily explained. It is of interest to note that **15E** which has *ortho*-methoxy on both rings A and B retained good induction activity (CD 2.5 μM) but was a weaker inducer than **3E** (CD 0.85 μM), indicating that the preference for an *ortho*-methoxy group may be restricted to ring A and not ring B.

Induction of NQO1 activity is known to proceed by at least two pathways, namely the arylhydrocarbon (AhR)/xenobiotic response element (XRE) signalling pathway or the Kelch-like ECH-associated protein 1 (Keap 1)/nuclear factor erythroid 2-related factor (Nrf2)/antioxidant response element (ARE) signalling pathway.[8–11] Agents that induce NQO1 via the AhR/XRE pathway also induce the activity of various phase I enzymes, notably the cytochrome P450 enzymes (CYP1A1, CYP1A2, CYP1B1). Agents that activate the Keap1/Nrf2/ARE pathway bring about the induction of only phase II enzymes like NQO1, GSTA2 and UGT1A6. It has been argued that induction of phase I enzymes is undesirable because the increased activity of some phase I enzymes like CYP450 can potentially activate polycyclic aromatic hydrocarbons to form ultimate carcinogens. We have shown earlier that **3E** which is the most potent NQO1 inducer among the resveratrol analogs failed to induce NQO1 in a mutant Hepa1c1c7 cell line (c1) that was defective in the expression of the CYP1A1 (aryl hydrocarbon hydroxylase) gene. This is shown in Figure 1 where different concentrations of **3E**

induced NQO1 activity in the wild type Hepa1c1c7 cells but not its c1 mutant.

As the CYP1A1 gene is part of the AhR gene battery and not the Nrf2 gene battery, it is deduced that **3E** induced NQO1 activity via the AhR/XRE pathway. Hence there is a concern that it may also induce CYP450 activity. Hence, the question is asked if **3E** induced CYP450 activity to the same degree as NQO1. This led us to determine the ability of **3E** to bring about the O-deethylation of 7-ethoxyresorufin in the 7-ethoxyresorufin deethylase (EROD) assay. EROD is the enzyme that mediates the O-deethylation of 7-ethoxyresorufin and it is widely used to assess the bioactivation capacity of CYP1A1.[12] **3E** was tested at 5 μM[13] which is equivalent to ca. 4 times its CD value for NQO1 induction. At this concentration, **3E** increased CYP1A1 activity by 1.2-fold. The level of NQO1 induction at the same concentration (4 × CD) was 4.9, which meant that **3E** selectively induced NQO1 by about fourfold compared to CYP1A1. The implication is that in spite of activating the less specific AhR/XRE pathway, differential induction of the various genes is possible. For **3E**, induction of the cytoprotective NQO1 exceeded that of the induction of CYP1A1 which plays a role in the activation of procarcinogens. The selectivity of **3E** for NQO1 induction over CYP1A1 induction may be related to its structural features since certain structural classes are associated with almost exclusive induction of phase 2 enzymes like NQO1 (with no induction of CYP1A1) while other compounds induced both phase 1 and 2 enzymes.[8,14]

Resveratrol and its methoxylated analogs were also evaluated for growth inhibitory effects on the Hepa 1c1c7 cells[15] (Table 1) A striking observation was the different structural features required for growth inhibition and NQO1 induction. Notably, the *Z* isomer was associated with more potent growth inhibitory effects, in contrast to its weak NQO1 induction activity. The compound with the most potent growth inhibitory effect was **10Z** (IC_{50} 0.03 μM) which had three methoxy groups (3,4,5-trimethoxy) on ring B. When tested at its IC_{50}, **10Z** failed to induce NQO1 by twofold and could not be tested at higher concentrations because of its adverse effect on the viability of the cells. Resveratrol had weak growth inhibitory effects on Hepa1c1c7 cells (IC_{50} >25 μM). The presence of OH groups on both rings A and B generally diminished growth inhibition effects and in this respect, is similar to the structure–activity trend observed for NQO1 induction.

To further confirm the growth inhibitory effects of resveratrol and its methoxylated analogs, the compounds were screened at 10 μM on two cancer cell lines, namely human breast cancer MCF7 cells and human colon HCT116 cells.[16] Of the 22 compounds tested, only nine compounds were found to reduce cell survival of both cell lines by more than 50% at this concentration. The IC_{50} of these compounds were then determined on the cancer cell lines (HCT116, MCF7) as well as the non-cancer human diploid fibroblasts derived from fetal lung (IMR90). The results are given in Table 2.

As anticipated, **10Z** had the greatest growth inhibitory effects, with IC_{50} values in the nanomolar range (MCF-7: 15 nM, HCT116: 20 nM). Its *E* isomer (**10E**) was at least a magnitude less potent than **10Z** and this stereoselective preference for the *Z* isomer was observed for other compounds. Thus, **8Z** was more potent than **8E**, while the remaining compounds shortlisted for IC_{50} determinations were all *Z* isomers. However, Cardile et al.[17] pointed out that the stereoselectivity was largely dependent on the cell lines used for evaluation. They noted that on the human melanoma cells (M14), the *E* isomer of resveratrol and two other methoxylated analogs (which coincidentally were **7E, 8E** in our study) were at least twice as potent as their corresponding Z isomers. We noted that **10E** had been extensively investigated by others.[5,18] Sale and co-workers proposed **10E** (referred to as DMU 212 in their paper) as a potentially useful chemopreventive agent because of its superior oral bioavailability compared to resveratrol[5] while Gosslau et al.[18] reported its ability to specifically activate the mitochondria-mediated apoptotic pathway as a means of inducing cell death. **10E** was also reported to have almost no growth inhibitory effect on normal cells although no IC_{50} values were reported. Here, we did not find **10E** to be outstanding in its ability to discriminate between cancer versus normal cells. As seen from Table 2, IC_{50} values of **10E** against HCT116/MCF-7 versus normal human diploid

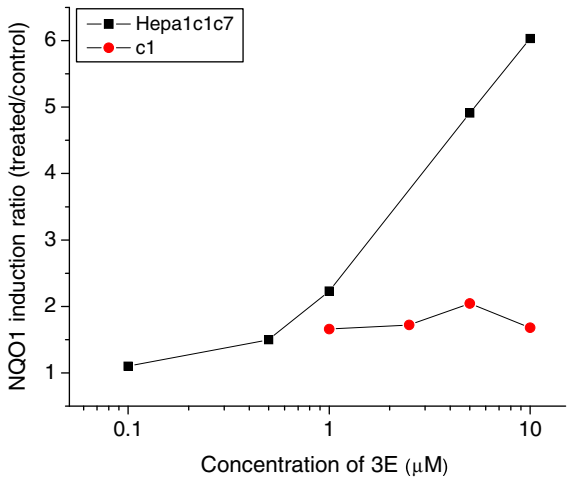
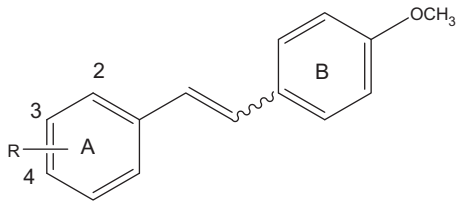


Figure 1. NQO1 induction activities of 3E on Hepa1c1c7 and c1 cells (NQO1 activity on wild type Hepa1c1c7 and mutant c1 cells (defective in expression of aryl hydrocarbon hydroxylase) were determined by the method described in Ref. 13. The wild type and mutant cells were purchased from ATCC (Rockville, MD)).

Table 2
Antiproliferative activity of methoxystilbenes on MCF7, HCT116 and IMR90 cell lines (IC_{50} and selectivity ratios)



Code	Ring A	IC_{50} (μM)[a]		
		MCF7	HCT116	IMR90
3Z	2-OCH_3	5.1 ± 0.1	5.9 ± 0.1	10.4 ± 0.6
		2.0	***1.8***	
4Z	3-OCH_3	4.4 ± 0.4	5.1 ± 0.1	9.5 ± 0.9
		2.2	***1.9***	
6Z	2,4-OCH_3	2.5 ± 0.1	2.3 ± 0.1	5.6 ± 0.4
		2.2	***2.4***	
7Z	3,4-OCH_3	2.4 ± 0.1	2.3 ± 0.1	7.0 ± 0.3
		2.9	***3.0***	
8E	3,5-OCH_3	1.1 ± 0.1	5.7 ± 0.6	2.1 ± 0.2
		1.9	***0.4***	
8Z	3,5-OCH_3	0.12 ± 0.03	0.22 ± 0.07	0.23 ± 0.02
		1.9	***1.0***	
10E	3,4,5-OCH_3	0.22 ± 0.03	0.53 ± 0.06	0.16 ± 0.04
		0.7	***0.3***	
10Z	3,4,5-OCH_3	0.015 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
		1.3	***1.0***	
14Z	3-OH, 4-OCH_3	4.2 ± 0.5	3.4 ± 0.5	6.0 ± 0.4
		1.4	***1.8***	
Doxorubicin		0.5 ± 0.1	0.09 ± 0.02	0.59 ± 0.1
		1.2	***6.6***	

[a] IC_{50} value is given as mean ± SD for $n = 3$ determinations. Values in bold and italics represent the selectivity ratio: $IC_{50\ IMR90}/IC_{50\ MCF7}$ or $IC_{50\ IMR90}/IC_{50\ HCT116}$.

embryonic lung fibroblast cells IMR90 were almost equivalent. A difference in the cell type and assay conditions (120 h incubation compared to 72 h in our experiments) may have accounted for this outcome. The less potent dimethoxy (**7Z**, IC_{50} ca. 2.4 μM) or monomethoxy (**6Z**, **4Z**, IC_{50} ca. 2.3–5.1 μM) analogs had better, although still modest (ca. 2–3-fold), selectivity ratios.

In conclusion, we have shown here that the methoxylation of the OH groups on resveratrol and the stereochemistry of the stilbenoid double bond have significant and contrasting effects on its ability to induce NQO1 activity and affect cell viability. The modest NQO1 induction activity of resveratrol was improved when the ring A OH groups were methoxylated. There was a noticeable preference for *ortho*-methoxy groups on ring A and an E configuration for the double bond. The most potent analog **3E** induced NQO1 to a greater extent than CYP1A1. Growth inhibitory effects were also improved by methoxylation of the ring A OH groups but with a preference for 2 or more methoxy groups at *meta* and *para* positions for good activity. The most potent analogs were the *Z* isomers but these compounds had only modest selectivity on cancer cells. There is continuing research interest in the potential of resveratrol for the treatment and prevention of many health related conditions like cancer[19], metabolic syndrome[20], neurodegeneration[21] and cardiovascular integrity.[22] In view of limitations relating to bioavailability and potency of resveratol, methoxylation of the scaffold is widely employed to investigate SAR. We have shown here that this approach can serve to enhance potency of some but not all activities. Thus, careful balancing of desired versus undesired effects is necessary in any structural modification exercise.

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15. Cell viability was determined on Hepa1c1c7 cells (American Type Culture Collection (Rockville, MD) using the microculture tetrazolium (MTT) assay. The cells were plated at a density of 10^4 cells per well in a 96-well plate and cultured for 24 h. The test compounds were prepared in DMSO and serially diluted with medium. Not more than 1% DMSO (final concentration) was present in each well. This concentration of DMSO was not cytotoxic to the Hepa1c1c7 cells and did not interfere with cell viability.
The test compounds were incubated with the cells for 48 h, after which 100 μl MTT (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 three separate occasions. The IC_{50} 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.
16. MCF7, HCT116 and IMR90 cells were purchased from American Type Culture Collection (Rockville MD). Culture media for growth of these cells were those recommended by ATCC. Cells were plated at the following densities 10000 cells/well (MCF7), 4000 cells/well (HCT116) 5000 cell/well (IMR90) and grown for 24 h in a humidified atmosphere of 5% CO_2, 37 °C. Cell viability was determined as described in Ref. 15.
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