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Newly constructed stable reporter cell lines for mechanistic studies on electrophile-responsive element-mediated gene expression reveal a role for flavonoid planarity

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Abbreviations:
BITC, benzyl isothiocyanate
DMSO, dimethyl sulfoxide
EpRE, electrophile-responsive element
FCS, foetal calf serum
GSH, glutathione
hNQO1, human NAD(P)H:quinone oxidoreductase 1
Keap1, Kelch-like erythroid cell-derived protein with CNC homology (ECH)-associating protein 1

ABSTRACT

The electrophile-responsive element (EpRE) is a transcriptional enhancer involved in cancer-chemoprotective gene expression modulation by certain food components. Two stably transfected luciferase reporter cell lines were developed, EpRE(hNQO1)-LUX and EpRE(mGST-Ya)-LUX, based on EpRE sequences from the human NAD(P)H:quinone oxidoreductase (hNQO1) and the mouse glutathione-S-transferase Ya (mGST-Ya) gene, containing one and two tandem EpRE core sequences, respectively. The standard inducer *tert*-butylhydroquinone (tBHQ), the electrophile benzyl isothiocyanate (BITC), and the antioxidant flavonoid quercetin were found to induce luciferase expression, thereby validating these newly developed reporter cell lines. For tBHQ and BITC, but not for quercetin, higher maximum luciferase induction was found under control of the mGST-Ya EpRE as compared to the hNQO1 EpRE, pointing at different induction mechanisms. Furthermore, we investigated the structure-activity relationship for induction of luciferase expression by flavonoids in EpRE(mGST-Ya)-LUX cells, and also the relation between luciferase induction and flavonoid antioxidant potency. Five different flavonoids with a planar molecular structure were found to induce various levels of luciferase activity, whereas taxifolin, a non-planar flavonoid, did not induce luciferase activity. This suggests that a stereospecific molecular interaction may be important for EpRE-mediated gene activation, possibly with Keap1, a regulator of EpRE-controlled transcription, or with another effector or receptor protein. No consistent relation between luciferase induction level and flavonoid antioxidant potential was observed. Altogether, these results point to differences in induction mechanism between the various chemoprotective compounds tested. The newly developed stably transfected reporter cell lines provide a validated tool for future screening and mechanistic studies of EpRE-mediated gene transcription.

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mGST-Ya, mouse glutathione S-transferase Ya
Nrf2, nuclear factor erythroid 2-related factor 2
PKC, protein kinase C
ROS, reactive oxygen species
tBHQ, *tert*-butylhydroquinone

1. Introduction

Diet has been identified as an important modulating factor of cancer risk, in particular of colorectal cancer [1]. The presence of carcinogenic as well as anticarcinogenic components in the human diet has been described [2–4]. The latter, so-called chemopreventive food constituents comprise a wide range of structurally diverse compounds such as flavonoids, indole-3-carbinol and its acid condensation products, carotenoids and certain other terpenoids, and glucosinolates and their isothiocyanate derivatives [3,4]. The cancer-preventive properties of fruits and vegetables, known to contain these anticarcinogenic components, have been demonstrated in experimental studies with laboratory animals [5], and in some human epidemiological studies, [6] although not consistently [7]. A common mechanism of action of these chemopreventive compounds is their ability to induce the activity of certain phase II biotransformation enzymes, for example NAD(P)H:-quinone oxidoreductase (NQO) [8], γ-glutamylcysteine synthetase [9], and certain glutathione S-transferase (GST) isoenzymes, such as GST-μ and GST-π [10] leading to more efficient detoxification of reactive genotoxic substances and relief of oxidative stress. This increased activity is regulated at the transcriptional level [11]. When studying the regulatory regions of the induced genes, a *cis*-acting enhancer element mediating the induction was identified [12,13]. This regulatory sequence was initially called the antioxidant-responsive element (ARE) [13–15], because of the antioxidant properties of some agents activating gene transcription through this element. Other studies observed a relation between the potency of activators of ARE-controlled transcription and their electrophilic nature [16,17] and therefore the element was subsequently also referred to as electrophile-responsive element (EpRE) [12]. The core sequence initially identified to be important for the EpRE function was determined by mutation analysis as RTGACnnnGC [13]. Later, several extensions of the original core sequence with additional functional nucleotides were reported [18–20] resulting in the definition of a consensus EpRE sequence by Wasserman and Fahl (Table 1). The nuclear factor erythroid 2-related factor 2 (Nrf2) is considered the major transcription factor involved in both constitutive and inducible expression of EpRE-regulated genes, as has been well documented in numerous in vitro and in vivo studies [9,21]. Currently, approximately 230 genes have been identified as being regulated by EpRE-mediated mechanisms [22].

Many investigators have used transient transfection, mostly with luciferase reporter gene constructs, to study EpRE-mediated gene expression. This approach however, has the disadvantage that it requires the repetitive laborious generation of a transfected cell population for every single assay, which hampers cost-effectiveness and throughput. Therefore, development of an appropriate continuous reporter cell line by stable transfection would be preferable.

The objective of the present study was to create and validate stable reporter cell lines using luciferase expression as a reporter of EpRE-mediated gene transcription activation. To account for the influence of structural differences in EpRE enhancer elements, two EpRE variants as described by Wasserman and Fahl [15] were utilized. A class II EpRE was used derived from the regulatory region of the human NAD(P)H:quinone oxidoreductase gene, which contains only one EpRE core sequence [15]; and a class III EpRE, derived from

Table 1 – Comparison of the mouse GST-Ya and human NQO1 EpRE elements from this study with reported EpRE core and consensus sequences

EpRE element	Nucleotide sequence [15]
Core	**RTGAC**nnn**GC**
Consensus	**TMA** nn**RTGAYnnnGC**Rwwww
GST-Ya, mouse	GTAGCT **TGG** AA**ATGAC**ATT**GC** **TAA** TG**GTGAC**AAA**GC**AACTTTA
NQO1, human	AG **TCA** CA**GTGAC**TCA**GC**AGAATC

Characteristic sequence hallmarks are indicated in bold. The so-called TMA box has been framed. M: A or C; R: A or G; Y: C or T; w: A or T; n: any nucleotide.

the regulatory region of the mouse glutathione-S-transferase (GST-Ya) gene, which has a second core-like sequence in tandem to the primary core [15]. These two EpRE variants also differ with respect to the nucleotide sequence flanking the core sequence, reported to influence the level of transcription activation [14–16,23]. The newly created reporter cell lines were validated using a set of model EpRE-mediated transcription inducers. Subsequently these cell lines were utilized to characterize the potential to induce EpRE-controlled gene transcription of a series of flavonoids, biofunctional secondary plant metabolites occurring in food, of interest because of their claimed anticarcinogenic potential [3]. The results are discussed in relation to the antioxidant and structural properties of the tested flavonoids, and reveal that a planar, aromatic C-ring is an important determinant of their activity as inducers of EpRE-mediated gene transcription.

2. Materials and methods

2.1. Chemicals and materials

Benzyl isothiocyanate (BITC) and *tert*-butylhydroquinone (tBHQ) were purchased from Sigma–Aldrich, taxifolin from MP Biomedicals (Amsterdam, The Netherlands), luteolin from Indofine Chemical Company (Hillsborough, NJ, USA), kaempferol from Extrasynthese (Genay, France), fisetin hydrate from Sigma–Aldrich (Zwijndrecht, The Netherlands), myricetin and quercetin from Acros Organics (Landsmeer, The Netherlands). All components tested were dissolved in DMSO (Acros Organics, Landsmeer, The Netherlands).

2.2. Cell culture

All cell culture reagents were purchased form Gibco (Invitrogen, Breda, The Netherlands). The mouse liver hepatoma cell line Hepa-1c1c7 was a kind gift from Dr. M.S. Denison, (University of California, Davis, CA, USA) and cultivated in the alpha modification of minimum essential medium (α-MEM), supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin and 100 μg/ml streptomycin. The human hepatoblastoma cell line (HepG2) was obtained from the European Collection of Cell Cultures (ECACC) (Sigma–Aldrich, Zwijndrecht, The Netherlands) and cultivated in Dulbecco's modified Eagle medium (DMEM)/F-12 Nutrient Mixture (F12) (1:1) containing glutamine, 15 mM Hepes, and pyridoxine, supplemented with 10% FCS, 100 IU/ml penicillin and 100 μg/ml streptomycin. Stably transfected cells were selected by cultivating in medium supplemented with 0.5 mg/ml of the antibiotic G418 (Duchefa Biochemie, Haarlem, The Netherlands). Cells were grown at 37 °C in a humid atmosphere containing 5% (v/v) CO_2.

2.3. Construction of EpRE-controlled luciferase reporter plasmids

The plasmid pGL3-basic, containing a promoterless enhanced luciferase reporter gene (Luc+), was purchased from Promega (Leiden, The Netherlands). An oligonucleotide containing a TATA box and an initiator (Inr) element [24], was cloned into the *Eco*RI and *Bam*HI site of the multiple cloning site of vector pSP72 (Promega, Leiden, The Netherlands). The constructed plasmid was digested with *Hind*III en *Bgl*II and the fragment containing the TATA/Inr sequence was subcloned into the *Hind*III and *Bgl*II site of pGL3-basic to obtain pGL3-TATA/Inr-Basic. Double stranded oligonucleotides were synthesized containing the EpRE enhancer element from the regulatory region of the mouse GST-Ya gene between −754 and −714 (5′-*TCGAG*TAGCTTG-GAAATGACAT TGCTAATGGTGACAAAGCAACTTT*A*-′3) [12,15,18], or of the human NQO1 gene, between −470 and −448 (5′-*TCGAG*TCACAGTGACTCAGCAGAATC*A*-′3) [14]. Both EpRE sequences were extended (italics) to create 5′-terminal *Xho*I and 3′-terminal *Bgl*II restriction sites (underlined). These oligonucleotides were annealed with their complement and inserted into the pGL3-TATA/Inr-Basic vector upstream of the TATA/Inr sequence. The resulting constructs containing the EpRE of the human NQO1 gene, and the EpRE of the mouse GST-Ya gene, were respectively designated pTI(hNQO1-EpRE)Luc+ and pTI(mGST-Ya-EpRE)Luc+. Plasmid DNA was purified using the Quantum Prep Plasmid Maxiprep Kit® (Bio-Rad Laboratories, Veenendaal, The Netherlands). The correct orientation and sequence of the oligonucleotides inserted into the reporter vector was confirmed by nucleotide sequencing (Baseclear, Leiden, The Netherlands).

2.4. Transient transfections and luciferase assays

Cells at 50–70% confluence were co-transfected with the vectors pTI(mGST-Ya-EpRE)Luc+ or pTI(hNQO1-EpRE)Luc+ and pRL-SV40 (Promega, Leiden, The Netherlands) as an internal standard for transfection efficiency, using a standard calcium phosphate-DNA co-precipitation transfection method [25]. The transfection medium was replaced by culture medium after 6 h. The next day cells were treated with the standard inducer *tert*-butylhydroquinone (tBHQ) [15,16] to test different concentrations and exposure times. Cells were washed with PBS, harvested and homogenized using Passive Lysis Buffer® (Promega, Leiden, The Netherlands), and cell lysates were clarified by centrifugation. Firefly and *Renilla* luciferase activity were measured in the supernatants using the Dual Luciferase Reporter Assay System® (Promega, Leiden, The Netherlands) and a Labsystems Luminoskan RS® luminometer (Thermo Electron, Breda, The Netherlands). Means and standard deviations were based on three independent replicates of cell transfection experiments and subsequent exposure.

2.5. Construction of stable reporter cell lines

To create the corresponding stably transfected Hepa-1c1c7 reporter cell lines, the cells were plated at 50–70% confluence and transfected with the luciferase reporter constructs pTI(hNQO1-EpRE)Luc+ or pTI(mGST-Ya-EpRE)Luc+ using a standard calcium phosphate-DNA co-precipitation transfection method [25]. The cells were co-transfected with pSV2-*neo* (Clontech, Westburg, Leusden, The Netherlands), which confers G418 resistance, to enable selection of successfully transfected cells. The next day the transfected cells were trypsinized, and plated again in culture medium containing 0.5 mg/ml of the antibiotic G418 (Duchefa Biochemie, Haarlem, The Netherlands) in a 30 times lower density to allow the formation of clonal colonies. The G418-resistant clones were

picked and propagated in medium containing 0.5 mg/ml of the antibiotic G418. The clone showing the highest absolute luciferase expression level in combination with the highest maximal luciferase induction factor, when exposed to the standard inducer tBHQ, was selected for use in further studies. The Hepa-1c1c7 clonal cell lines stably transfected with pTI(hNQO1-EpRE)Luc+ and pTI(mGST-Ya-EpRE)Luc+ were designated EpRE(hNQO1)-LUX and EpRE(mGST-Ya)-LUX, respectively.

2.6. Reporter gene assays using stable EpRE-LUX reporter cell lines

EpRE(hNQO1)-LUX cells and EpRE(mGST-Ya)-LUX cells were propagated as described above. To investigate the effect of tBHQ, benzyl isothiocyanate (BITC) and different flavonoids on EpRE-mediated gene expression the cells were seeded in a concentration of 2×10^4 cells/well in a 96-wells view-plate (Amersham Biosciences, GE Healthcare, Roosendaal, The Netherlands) and incubated for 24 h to allow the formation of a confluent monolayer. Subsequently, the medium was replaced by medium without FCS or antibiotics, containing the test compound in a concentration range from 0.3 to 100 μM. The compounds were added dissolved in DMSO, and the final DMSO concentration in the cell culture medium was kept at 0.5% (v/v). After exposure for 24 h cells were washed with 0.5 M PBS (phosphate-buffered saline, pH 7.4), harvested and homogenized in a hypotonic lysis buffer (10 mM Tris, 2 mM DTT and 2 mM *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetra-acetic acid mono-hydrate (CDTA), pH 7.8). Plates were placed on ice for 15 min to allow swelling of the cells and subsequently frozen at −80 °C for at least 30 min to lyse the cells. Subsequently, 100 μl Flash Mix (20 mM tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2{\cdot}5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA·$2H_2O$, 2 mM DTT, 0.47 mM D-luciferin, 5 mM ATP, pH 7.8) was added and luciferase activity was measured for 2 s using a Labsystems Luminoskan RS® luminometer (Thermo Electron, Breda, The Netherlands). After measurement, the remaining luciferase activity was extinguished by adding 50 μl 0.2 M NaOH to the reaction mixture. Luciferase expression data were based on independent measurements of six different wells and presented as the average induction factor compared to the unexposed control ± the standard error of the mean. All incubations with flavonoids were performed in medium containing 1 mM Vitamin C to prevent auto-oxidation of the flavonoids in the medium [26]. This concentration of Vitamin C did not induce EpRE activation by itself (data not shown).

3. Results

3.1. Construction of luciferase reporter plasmids

Two different luciferase reporter constructs were generated. The first construct, designated pTI(mGST-Ya-EpRE)Luc+, carried two tandem EpRE enhancer core sequences from the upstream regulatory region of the mouse glutathione S-transferase-Ya (GST-Ya) gene as the regulatory sequence controlling luciferase expression [16]. The second construct was similar, but contained an EpRE with a single core sequence from the upstream regulatory region of the human NAD(P)H:quinone oxidoreductase 1 (hNQO1) gene [15], and was named pTI(hNQO1-EpRE)Luc+. Both constructs also differ substantially from each other with regard to the nucleotides flanking the EpRE core sequence (Table 1). In a first series of transient transfection experiments into mouse Hepa-1c1c7 and human HepG2 cells the functionality of the constructed reporter plasmids was tested. The transiently transfected cells were exposed during 24 h to 90 μM of the established inducer tBHQ, which has been reported to produce maximal EpRE-mediated transcription activation in mouse Hepa-1c1c7 cells [14]. The results presented in Table 2 demonstrate that the pTI(mGST-Ya-EpRE)Luc+ reporter plasmid, carrying the mouse GST-Ya EpRE, mediates significant tBHQ-inducible luciferase expression when transfected into homologous mouse Hepa-1c1c7 cells. Accordingly, pTI(hNQO1-EpRE)Luc+, carrying the NQO1-EpRE of human origin, showed significant tBHQ-inducible luciferase expression upon transfection into homologous human HepG2 hepatoma cells (Table 2). The heterologous combination of pTI(mGST-Ya-EpRE)Luc+ transfected into human HepG2 cells was found to give rise to an even higher level of tBHQ-induced luciferase expression than upon transfection into the homologous mouse Hepa-1c1c7 cells. On the other hand, the EpRE of human origin carried by pTI(hNQO1-EpRE)Luc+ turned out to be only slightly active upon transfection into the mouse Hepa-1c1c7 cells, since only

Table 2 – Induction of luciferase expression in Hepa-1c1c7 and HepG2 cells transiently transfected with the indicated reporter plasmid upon exposure to *tert*-butylhydroquinone (tBHQ)

Origin of EpRE	Exposure time (h)	Luciferase induction factor [95% confidence interval ($p < 0.05$)]		
		[tBHQ]		
		90 μM		180 μM
		Hepa-1c1c7	HepG2	HepG2
Mouse GST-Ya	6	–	2.1 [1.5–2.7]	–
	12	–	4.4 [3.2–5.5]	–
	24	2.5 [2.0–3.0]	5.1 [3.8–6.3]	6.4 [5.1–7.7]
Human NQO1	6	–	1.2 [1.1–1.4]	–
	12	–	1.9 [1.7–2.1]	–
	24	1.1 [1.0–1.2]	2.2 [1.5–2.9]	2.3 [1.5–2.9]

marginal luciferase induction was observed at 90 μM tBHQ (Table 2).

Because the highest levels of luciferase induction were observed in HepG2 cells for both pTI(mGST-Ya-EpRE)Luc+ and pTI(hNQO1-EpRE)Luc+, this cell type was selected to subsequently study concentration and time dependency of the luciferase induction by tBHQ. With both EpRE variants the luciferase induction by 90 μM tBHQ in transiently transfected HepG2 cells was found to increase with exposure time. Luciferase expression did not appear to increase at higher concentrations of tBHQ (Table 2).

Altogether, these results provide evidence that the constructed plasmids confer EpRE-controlled luciferase expression upon transfection into cells supporting EpRE-mediated transcription activation.

3.2. *Construction of stable luciferase reporter cell lines for EpRE-mediated gene expression activation*

Since Hepa-1c1c7 cells have a doubling time of 15 h [27], which is much shorter than that of HepG2 cells (more than 29 h [28]), Hepa-1c1c7 cells were used to obtain a continuous reporter cell line featuring stable EpRE-mediated luciferase expression. Stable transfectants were generated by co-transfection of Hepa-1c1c7 cells with pSV2-*neo*, carrying a *neo*^{r} selection marker conferring resistance to the antibiotic G418, and pTI(mGST-Ya-EpRE)Luc+ or pTI(hNQO1-EpRE)-Luc+. The G418-resistant clones were isolated and the clone showing the highest maximal luciferase induction factor in combination with the highest luciferase expression level upon exposure to tBHQ was selected for further validation and application as a reporter cell line. The obtained cell line carrying the luciferase reporter under control of the mouse GST-Ya EpRE element was designated EpRE(mGST-Ya)-LUX and the cell line carrying the EpRE element from the human NQO1 regulatory region is referred to as EpRE(hNQO1)-LUX.

3.3. *Induction of luciferase activity in EpRE-LUX cells by established inducers of EpRE-controlled gene transcription*

The newly constructed reporter cell lines were validated by exposure during 24 h to a concentration range of tBHQ, the standard inducer of EpRE-controlled gene transcription, and two dietary compounds, benzyl isothiocyanate (BITC) and quercetin, as examples of established chemoprotectants and known inducers of EpRE-mediated gene expression [11,29]. In the EpRE(mGST-Ya)-LUX (Fig. 1A), as well as in the EpRE(hNQO1)-LUX-cells (Fig. 1B), all compounds tested showed a concentration-dependent luciferase induction up to a maximal level, which was different for the various compounds. The induction of luciferase in the created cell lines is consistent with the postulated EpRE-mediated nature of the response, validating the newly developed reporter gene assays.

The luciferase induction by the three inducers tested showed the same ranking order in both created cell lines and was highest after treatment with the dietary isothiocyanate BITC, followed by tBHQ, which produced a maximal induction of luciferase activity amounting to two third of that of BITC, while quercetin gave the lowest induction level (Fig. 1A and B).

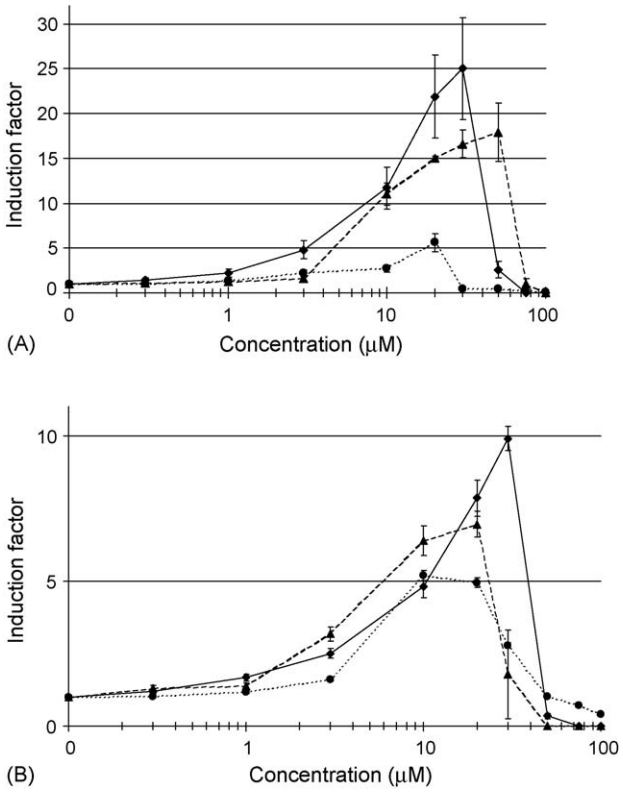
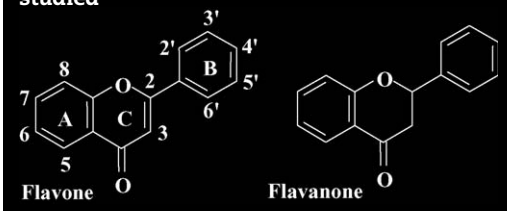


Fig. 1 – Luciferase induction in (A) EpRE(mGst-Ya)-LUX cells, and (B) EpRE(hNQO1)-LUX cells upon 24 h of exposure to the standard EpRE-mediated transcription activator *tert*-butylhydroquinone (tBHQ) (triangles) and two dietary compounds, the electrophilic compound benzyl isothiocyanate (BITC) (squares) and the antioxidant quercetin (dots). Data points represent the mean ± S.E.M. of six replicates.

In the EpRE(hNQO1)-LUX cell line quercetin reached almost the same maximum plateau of five- to six-fold luciferase induction as tBHQ (Fig. 1B), whereas in the EpRE(mGST-Ya)-LUX cell line, quercetin also reached a five-fold induction, which is only a fraction, however, of the 18-fold induction by tBHQ (Fig. 1A). Both tBHQ and BITC gave different maximal responses in the two created EpRE-LUX cell lines. The EpRE(mGST-Ya)-LUX cell line showed approximately a 2.5 times higher luciferase induction after the treatment with BITC and tBHQ than the EpRE(hNQO1)-LUX cell line, but the ratio of the maximal induction by the two compounds was similar.

A decrease in induction factor of luciferase activity was found at higher concentrations with all tested chemoprotectants. This effect is probably due to cytotoxicity, since the integrity of the cell layer in the 96 wells test plate was found to be damaged upon visual inspection at concentrations of the tested dietary compounds higher than 50 μM. Moreover, these microscopic observations were consistent with the results from cell proliferation measurements in HepG2 cells, based on bromodeoxyuridine (BrdU) incorporation, and showing a decrease in cell proliferation rate in this concentration range (data not shown).

Table 3 – Molecular structure of the various flavonoids studied



Flavonoid	Class	Hydroxylation Pattern
Quercetin	Flavone	3,5,7,3′,4′
Fisetin	Flavone	3,7,3′,4′
Kaempferol	Flavone	3,5,7,4′
Luteolin	Flavone	5,7,3′,4′
Myricetin	Flavone	3,5,7,3′,4′,5′
Taxifolin	Flavanone	3,5,7,3′,4′

3.4. *Flavonoid structure-activity relationship for EpRE-mediated transcription activation*

Subsequently, the structure-activity relationship for the activation of EpRE-mediated gene transcription by flavonoids was studied using the EpRE(mGST-Ya)-LUX reporter cells. To identify the elements in the molecular structure of quercetin involved in the observed stimulation of EpRE-controlled gene transcription, the potency to induce luciferase expression of various flavonoids that differ from quercetin in one single structural element (Table 3) was determined.

All flavonoids tested, except for taxifolin, showed a dose-response curve comparable in nature to that of quercetin (Fig. 1) with a dose-dependent increase of the luciferase induction response up to 10–30 μM, followed by a decrease in response, apparently due to increasing cytotoxicity. The maximal induction factor reached by each flavonoid is shown in Fig. 2. Although fisetin lacks the 5-hydroxyl moiety on the A ring compared to quercetin, it reaches a slightly higher maximal luciferase induction level than quercetin (Fig. 2), suggesting that the 5-hydroxyl group on the A ring is not important for induction. Luteolin, kaempferol and myricetin were found to attain a comparable, but significantly lower maximal induction level than quercetin and fisetin (Fig. 2). Luteolin lacks the 3-hydroxyl group on the C ring compared to quercetin, while kaempferol lacks the 3′-hydroxyl group resulting in a phenolic instead of a catecholic B ring. Myricetin, shares the same aromatic core structure with quercetin but contains an additional hydroxyl group on the B ring at C-5′. Therefore, their observed lower potency could not clearly be attributed to a specific structural element. The most obvious structure-activity relationship that emerged from this study was the clear absence of luciferase induction potency observed for taxifolin as compared the other flavonoids tested. This seems to be associated with the distinct structural property of taxifolin having a non-planar aliphatic C-ring due to lack of the C2–C3 double bond. All other flavonoids tested possess the C2–C3 double bond, suggesting that a planar aromatic C-ring may be important for efficient EpRE-mediated transcription activation by flavonoids.

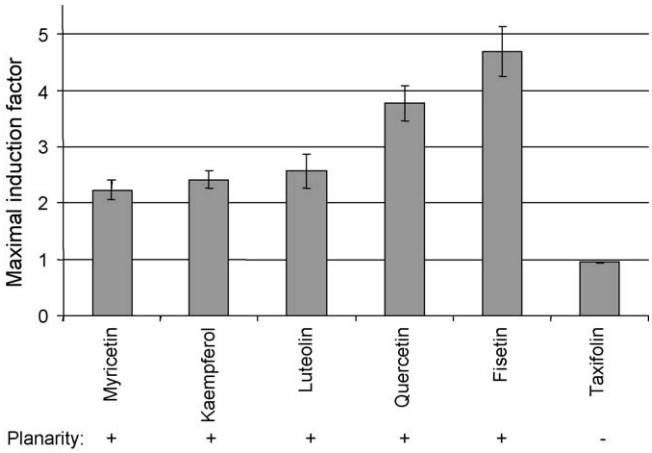


Fig. 2 – Maximal luciferase induction factor in EpRE(mGST-Ya)-LUX cells attained by various planar flavonoids and by the non-planar flavonoid taxifolin. EpRE(mGST-Ya)-LUX cells were exposed during 24 h to a concentration range of the indicated flavonoids and the maximal level of luciferase induction reached by each flavonoid was plotted. Maximal luciferase induction was observed at 10 μM for myricetin, and at 30 μM for kaempferol, luteolin, quercetin, and fisetin. For taxifolin the mean induction factor (0.95 ± 0.01) observed over the entire concentration range (0.3–50 μM) was plotted, since no significant response was observed at any of the concentrations tested. The positive control tBHQ reached a maximal induction factor of 22.9 ± 4.4 times at 30 μM (not shown).

Table 4 – Ranking order of antioxidant activity of the model flavonoids of the present study in different antioxidant assays in comparison to their ranking order with respect to EpRE-mediated gene transcription activation potency

Assay	Decreasing order of potency[a]	Reference
EpRE-LUX	1 > 2 > 3 = 4 = 5 > 6	
Antioxidant activity[b]		
TEAC	2 > 4 > 3 > 6 > 5	[55]
Ep/2	4 > 2 > 5 > 6 > 3	[56]
IC_{50} enzymatic	4 ≥ 2 = 3 = 1 > 5 > 6	[56]
IC_{50} non-enzymatic	5 > 2 ≥ 4 > 1 ≥ 3 > 6	[56]
Iron chelation	6 = 1 > 2 = 3 = 5	[56]
K_2	4 > 2 > 1 > 5 > 3 > 6 >	[57]
Reaction stoichiometry	4 > 1 > 2 > 3 > 6 > 5	[57]
Spin labeling	4 ≥ 2 > 5	[58]
IC_{50} DPPH	1 > 3 > 2 > 5 > 4	[59]
IC_{50} AAPH	3 > 5 > 2 > 1	[59]

[a] Maximal luciferase induction level reached by the flavonoid in EpRE(GST-Ya)-LUX cells, or antioxidant activity, respectively. 1: fisetin; 2: quercetin; 3: luteolin; 4: myricetin; 5: kaempferol; 6: taxifolin.

[b] For details on the antioxidant activity parameters the reader is referred to the respective references.

3.5. *Relation between flavonoid antioxidant activity and EpRE-mediated transcription-activating potency*

In addition to the relation between hydroxyl group substitution pattern, planarity, and the potency to induce EpRE-controlled luciferase expression, the relation with the antioxidant properties of the tested flavonoids was also studied. Numerous literature reports describe the antioxidant capacity of flavonoids (Table 4). As shown in Table 4 the ranking of the studied flavonoids according to antioxidant capacity was found to be highly dependent on the type of antioxidant assay used and the assay conditions applied. In most assays myricetin and quercetin were found to be the best antioxidants. But also kaempferol, taxifolin, fisetin and luteolin were found to be among the most potent antioxidants in particular assays. Since none of the potency rankings listed in Table 4 matches the ranking found for the capacity to generate a response in the EpRE(mGST-Ya)-LUX assay, it is concluded that there is no clear relationship between antioxidant properties of the tested flavonoids and the potential to induce EpRE-mediated gene expression.

4. Discussion

EpRE-mediated activation of gene expression is considered an important aspect of the cancer-preventive action of chemoprotective dietary compounds. The present study describes the generation and validation of two stable reporter cell lines based on different EpRE elements with one, respectively two EpRE core sequences as their main difference. All known activators of EpRE-controlled transcription tested here, including reducing antioxidants (exemplified by tBHQ and quercetin) and electrophilic compounds (BITC), dose-dependently induce luciferase activity in both constructed reporter cell lines EpRE(hNQO1)-LUX and EpRE(mGST-Ya)-LUX (Fig. 1A and B), thereby demonstrating the validity of the newly developed cell lines as assay systems to measure EpRE-mediated transcription activation. The developed assays allowed to establish a potency ranking for EpRE-mediated gene transcription activation within a group of structurally related flavonoids (Table 4), which exemplifies their applicability as a screening tool. The 96-well format and low costs of the assay would also allow high-throughput applications.

The human NQO1 EpRE used to construct the EpRE(hNQO1)-LUX reporter cell line contains only one copy of the EpRE consensus sequence (Table 1), whereas the mouse GST-Ya EpRE (Table 1), used to develop the EpRE(mGST-Ya)-LUX reporter cell line, contains one full consensus EpRE and one EpRE with a non-consensus TGG sequence at the TMA box position [15], which was nonetheless found to contribute to transcription activation [30]. Consistently, we observed a 2.5-fold higher maximal luciferase induction by BITC and tBHQ in the EpRE(mGST-Ya)-LUX as compared to the EpRE(hNQO1)-LUX cell line. In contrast, EpRE-mediated transcription activation by quercetin seems to be independent of the number of functional EpRE sequences present, since quercetin reached the same five-fold maximal induction factor in both EpRE-LUX cell lines. This may point to differences in the mechanism of EpRE-mediated transcription activation between quercetin on the one hand, and BITC and tBHQ on the other hand.

It has been clearly established that nuclear factor-erythroid 2-related factor 2 (Nrf2) is the key regulator of EpRE-mediated gene expression, which binds to the EpRE site as a heterodimer with a small Maf protein as the most probable dimerisation partner [31]. The activity of Nrf2 as a transcription factor is regulated by complex formation with a cysteine-rich protein, called Kelch-like erythroid cell-derived protein with CNC homology (ECH)-associating protein 1 (Keap1) [32,33].

Different Nrf2 activation mechanisms have been suggested to result in the release of Nrf2 by Keap1 and accumulation of free Nrf2 in the nucleus [23,33,34]. Cysteine adduct or intermolecular disulfide bond formation in Keap1 [32,33,35,36], phosphorylation of Nrf-2 by PKC or other kinases [23,34,37], the redox status of a sulfhydryl group in the DNA-binding domain of Nrf2 [38,39], the presence of functional thioredoxin in the nucleus [39], and the glutathione redox system in the cytoplasm [39] were all found to be modulating factors contributing to the complex regulation of Nrf2 activity.

Although implied by the name antioxidant-responsive element, there seems to be no consistent relation between the antioxidant activity of a compound and its potency to activate gene transcription through an EpRE, as demonstrated by the group of flavonoid compounds investigated in this study (Table 4). This suggests that EpRE-mediated activation of gene transcription by flavonoids involves other biochemical properties of flavonoids than their antioxidant action. That the release of transcription factor Nrf2 from Keap1 is thought to involve oxidation of cysteine sulfhydryl groups of Keap1 [33,35] is consistent with this conclusion, since cysteine oxidation would require pro-oxidant rather than anti-oxidant action. Furthermore, a role for other mechanisms than antioxidant action is also supported by our observation that absence of the C2–C3 double bond in the C-ring, as in taxifolin, completely abolishes the ability of the flavonoid to stimulate gene expression via the EpRE (Fig. 2). It has been reported [40] that the absence of the C2-C3 double bond results in a decreased tendency of a catechol B-ring flavonoid to become oxidized and cause oxidative stress by co-oxidation of cellular anti-oxidants such as ascorbate and glutathione. However, taxifolin still causes some co-oxidation of cellular anti-oxidants [41] implying that the redox properties of taxifolin do not provide a likely explanation for its complete inability to induce EpRE-mediated gene transcription, as compared to the other flavonoids tested. Another consequence of the absence of the C2–C3 double bond is loss of planarity of the flavonoid molecule, which presents an exclusive difference between taxifolin and the other flavonoids tested. Therefore, the results obtained in the EpRE(mGST-Ya)-LUX cell line suggest that a specific stereochemical interaction, possibly with Keap1, or with another effector or receptor protein, may explain the distinctive responsiveness to the planar flavonoids in contrast to the lack of response to the non-planar taxifolin. This observation is consistent with the conclusion of Uda et al. [8] that the C2–C3 double bond is necessary for the flavonoid to induce NQO1 expression, and also with the observation of Miranda et al. [42] that the non-planar flavonoid naringenin is not an NQO1 inducer. On the other hand, our results may seem contradictory to literature reports that some other non-planar

flavonoids, like pinostrobin and its metabolites, are potent inducers of NQO1 gene expression [43]. It was shown, however, that the NQO1 induction mechanism activated by these non-planar flavonoids requires an active Ah receptor, suggesting that the xenobiotic response element (XRE) also present in the NQO1 regulatory region may be involved instead of the EpRE element [43].

This is to our knowledge the first experimental observation in an EpRE-regulated reporter gene system suggesting that, in addition to electrophilic [16], or Michael reaction acceptor [44] potential, the stereochemical properties of certain compounds may also present an important determinant of their potency as an EpRE-mediated transcription activator. Thus, our reporter gene studies suggest the existence of an as yet unrecognized mechanistic aspect of EpRE-mediated gene transcription activation, involving a stereospecific interaction, possibly with Keap1 itself, or with another effector or receptor protein. The occurrence of electrophile-specific patterns of adduct formation within the Keap1 protein may also reflect differences in accessibility of the various Keap1 domains dependent on the molecular structure of the electrophile [45].

Current knowledge on the complex mechanism of EpRE-mediated transcription activation is consistent with the concept of multiple activation routes that may be activated in singular or in parallel, dependent on the chemical nature of the inducer. The higher-fold EpRE-mediated induction levels observed for tBHQ and BITC as compared to quercetin, for example, are likely due to the capacity of the two former inducers to affect additional activation routes as compared to quercetin. Some studies indicated PKC-dependent phosphorylation of Nrf2 to be involved in induction of EpRE-mediated gene expression by tBHQ [34,46,47], but other studies showed that PKC is not involved [48,49], or that other protein kinases are involved [49–51]. Furthermore, tBHQ and quercetin and their quinone metabolites may engage in redox cycling and induce oxidative stress [52]. tBHQ was also found to influence GSH levels, and the glutathione to glutathione disulfide ratio, but not the ratio of reduced to oxidized thioredoxin [39]. In addition, we hypothesize on the basis of our structure-activity analysis that activation of EpRE-regulated transcription by planar flavonoids may involve stereospecific interaction, possibly with Keap1 or with another receptor or effector protein. The electrophilic isothiocyanates, such as BITC, have been reported to induce cysteine adduct and disulfide bond formation within Keap1 [32,35,53], and to cause GSH depletion [39,54].

Altogether, it remains unclear which of these possible mechanisms are actually involved in producing the response observed in our reporter cell lines, and what their relative contribution is. However, the study of the flavonoid structure-activity relationship presented here provides evidence that stereochemical properties are an as yet unrecognized important determinant of the potency of certain inducers of EpRE-mediated transcription activation, and demonstrates that the constructed stable reporter cell lines provide a useful tool for both screening and mechanistic studies. Extensive and detailed experimental analysis using selective inhibition of each pathway of Nrf2 activation by biochemical or genetic methods is needed to achieve comprehensive mechanistic understanding of EpRE-mediated gene transcription activation by dietary electrophiles and antioxidants.

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