



In vitro and *in vivo* interplay
between
NAD(P)H: quinone oxidoreductase 1
and flavonoids

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Yuen Yee Hilz

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Abstract

Flavonoids are naturally occurring, health-promoting, bioactive compounds, omnipresent in the human diet. The protective effect of these phytochemicals is accomplished for an important part by modulating the activity of enzyme systems responsible for deactivation of chemical carcinogens, such as NAD(P)H: quinone oxidoreductase 1 (NQO1). Several flavonoids act as NQO1 inducers by increasing the NQO1 gene expression level through the electrophile-responsive element (EpRE). On the other hand certain flavonoids are efficient inhibitors of the NQO1 enzyme activity *in vitro*. The objective of this thesis is to elucidate the complex interplay between flavonoids and NQO1. First, inhibition of NQO1 by flavonoids, pointing at a mechanism contradicting the proven beneficial properties of these natural compounds was studied. Kinetic and molecular dynamics studies were conducted and a method to monitor NQO1 activity in living cells was developed. These studies revealed that although flavonoids possess the potential to inhibit NQO1 activity, inhibition of NQO1 is not likely to happen in cellular systems due to intracellular physiological conditions.

Furthermore, the mechanism by which flavonoids are able to induce the EpRE-mediated expression of NQO1 was studied. Reporter gene assays elucidated that upstream XRE-mediated gene expression is not necessary to induce EpRE-mediated gene expression and quantum-mechanical calculations revealed that flavonoids with a higher intrinsic potential to generate oxidative stress and redox cycling, are the most potent inducers of NQO1. Radioactive binding studies showed Keap1 modification by the flavonoid quercetin, resulting in switching on of EpRE-mediated gene transcription activation. In addition, *in vivo* metabolites of quercetin were studied on their ability to induce EpRE-mediated gene expression. The results show, that, although quercetin-derived glucuronides are the major metabolites present in the systemic circulation, the deglucuronidated parent compound and its methylated derivatives are the active compounds responsible for the beneficial EpRE-mediated gene expression effects.

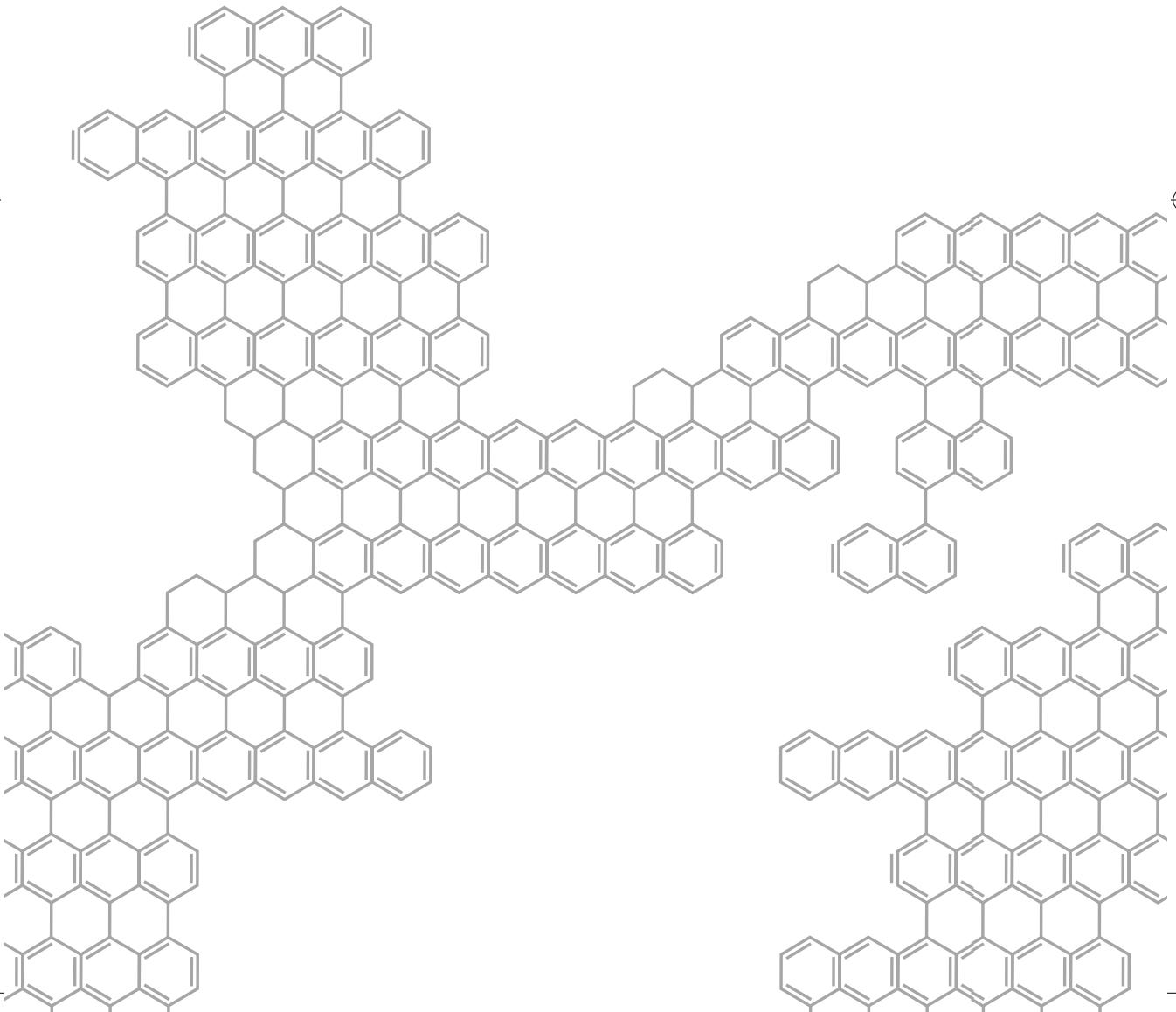
Overall, the studies presented in this thesis provide insight in the complex interplay between NQO1 and flavonoids on the protein as well as on the gene expression level.

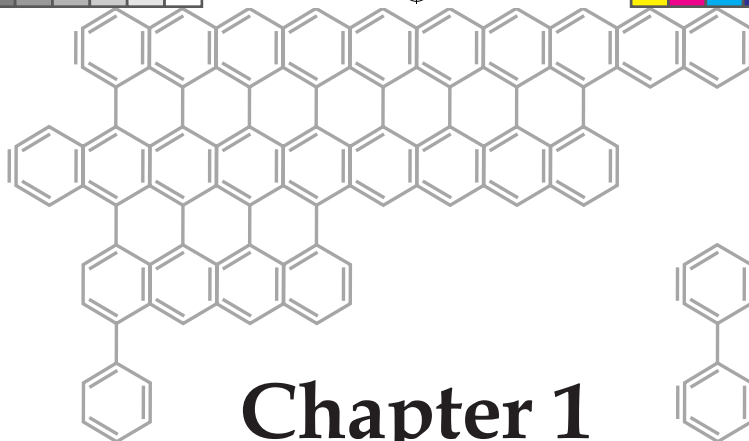




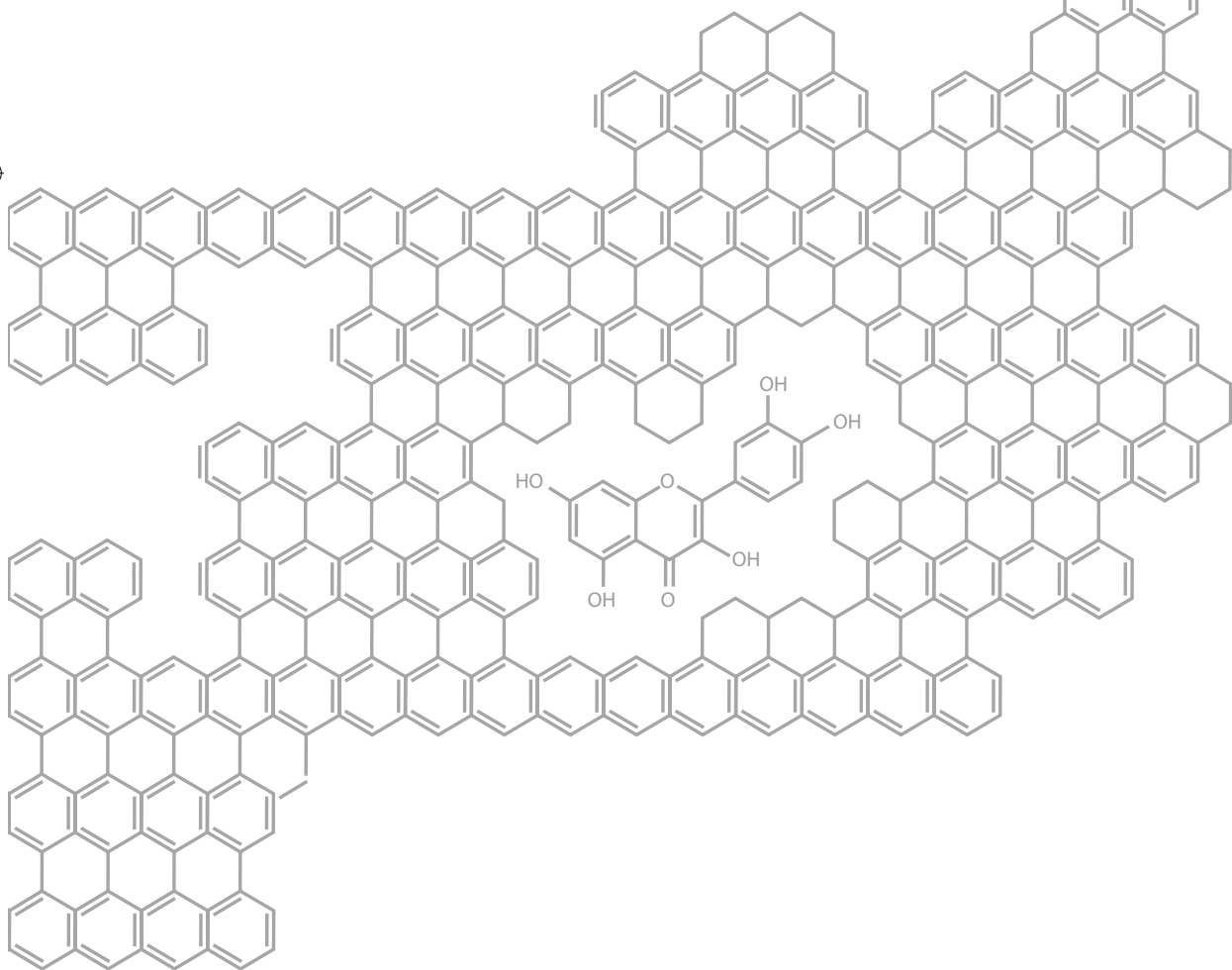
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General Introduction



1. Flavonoids

Flavonoids are phytochemicals belonging to the group of the polyphenols. These aromatic compounds are secondary metabolites produced in most plants, contributing to the plants growth, development and defence against micro-organisms. The beneficial health effects of fruit and vegetable consumption have partly been attributed to the bioactive properties of flavonoids. The average intake of these phytonutrients is estimated to be about 1 g/day, which presents about two thirds of the total intake of polyphenols (Scalbert and Williamson 2000). The precise amount and composition of flavonoid consumption varies widely due to dietary habits, especially the consumers' intake of fruits and vegetables, and the possible use of flavonoids as dietary supplements (capsules contain in average 100–200 mg flavonoids) (Manach *et al.* 2004).

Flavonoids consist of six major classes based on specific structural differences: flavanols, flavones, flavanones, catechins, anthocyanidins and isoflavones. The basic flavonoid skeleton, and the six major classes are depicted in Figure 1.1.

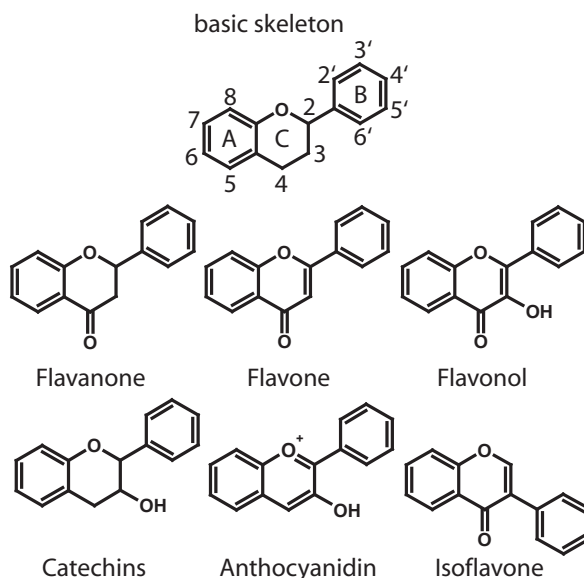


Figure 1.1: Structures of 6 major classes of flavonoids.

Flavonoids comprise a basic structure of 15 carbon atoms with two aromatic rings (A and B, Figure 1.1) connected through a 3 carbon chain, which make up a third ring (C ring, Fig. 1.1). The type of carbon skeleton, contributes to the chemical diversity of these compounds. Flavonoids within a class vary with respect to the number, position and conjugation pattern of their hydroxyl substituents.

Due to the variation in number and location of hydroxy and/or methoxy substituents more than 8000 different flavonoid structures have been identified (Pietta 2000).

1.1. Bioavailability of flavonoids

Flavonoids in plants are present as β -glycosides of the aglycones. This conjugation can be with a variety of sugars, including D-glucose, L rhamnose, glucorhamnose, galactose, lignin, and arabinose. Figure 1.2 shows a schematic overview of the metabolism of flavonoid glycosides.

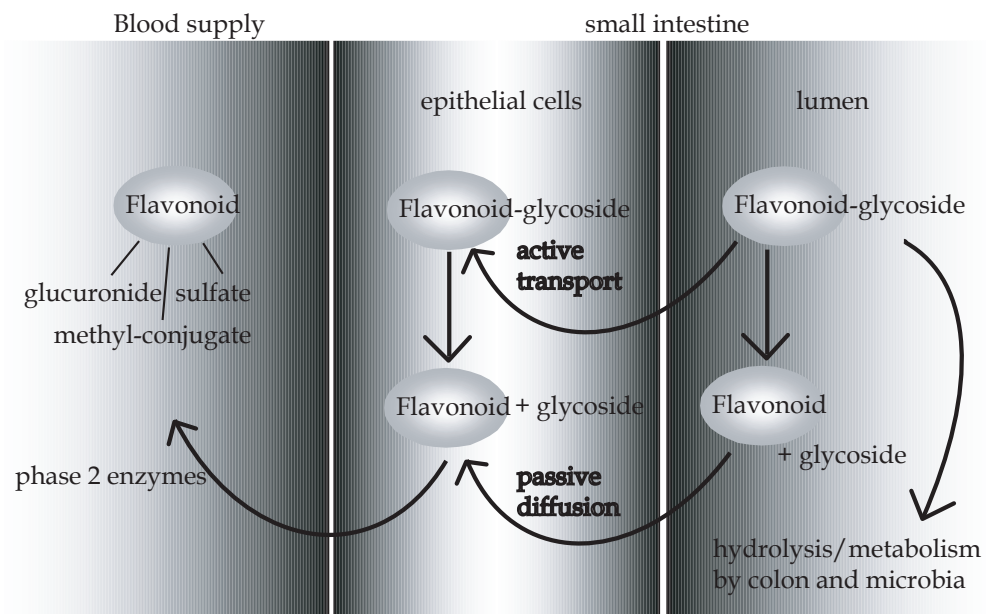


Figure 1.2: Metabolism of flavonoids upon ingestion

Upon oral intake, flavonoids are absorbed from the gastrointestinal tract. Upon ingestion, flavonoids are believed to pass through the small intestine, which can act as an effective absorption site for flavonoid glycosides (Murota and Terao 2003). Furthermore, specific glycosidases on the enterocytes may contribute to hydrolysis followed by uptake of the aglycones into the enterocytes (Day *et al.* 1998). Upon passage through the small intestine, the flavonoid glycosides can enter the cecum and the colon, where they are hydrolyzed to the aglycone by enterobacteria (Walle 2004). The flavonoid aglycones are subject to O-methylation, glucuronidation and/or sulfation in the liver (van der Woude *et al.* 2004). This presents a metabolic process common to many xenobiotics to restrict their potential toxic effects and facilitates their biliary and urinary elimination by increasing their hydrophilicity. These conjugation mechanisms are highly efficient, and aglycones are generally either

absent in blood or present in low concentrations after consumption of dietary doses. Only a small part of the flavonoid metabolites is transported to the blood stream and circulates, with flavonoid glucuronides being the major metabolites present in the systemic circulation (de Boer *et al.* 2005; Kanazawa *et al.* 2006). The plasma concentrations of total flavonoid metabolites range from 0 to 4 $\mu\text{mol/L}$ upon an intake of 50 mg aglycone equivalents (Manach *et al.* 2005).

1.2. Bioactivity of flavonoids

Epidemiological studies have shown that flavonoid intake is associated with reduced risk of many chronic diseases including coronary heart disease, cancer, diabetes, arthritis and osteoporosis. Flavonoids have anti-inflammatory, anti-allergic, and antiviral activities. Beside these activities, flavonoids are also known to be cytostatic, apoptotic, anti-thrombotic and anti-estrogenic (Scalbert *et al.* 2005). Many of the biological actions of flavonoids have been attributed to their antioxidant properties, either through their reducing capacities per se or through their possible influences on the intracellular redox status (Rice-Evans 2001). Oxidants are constantly generated for essential biological functions. However, an excess generation or an imbalance between oxidants and antioxidants can produce a pathophysiological condition known as oxidative stress. Flavonoids have powerful antioxidant activities *in vitro*, being able to scavenge reactive oxygen species (ROS), including hydroxyl, peroxy and superoxide radicals (Halliwell *et al.* 2005). However, flavonoids can also display pro-oxidant activity, after donating electrons either by antioxidant action, enzymatic oxidation or autooxidation (Awad *et al.* 2000). Flavonoids with a catechol moiety have the potential to oxidize to quinones or semiquinones resulting in redox cycling and ROS production as well as in thiol, DNA and protein alkylation (Fig. 1.3) (van der Woude *et al.* 2005a).

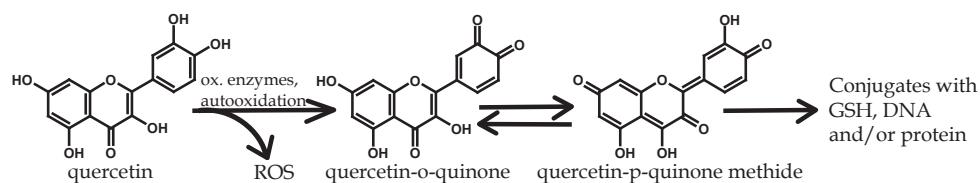


Figure 1.3: Pro-oxidant activity of quercetin

The pro-oxidant action of flavonoids is generally considered as unfavorable. However, accumulating evidence exists that part of the beneficial effects of flavonoids are based on their pro-oxidant rather than their antioxidant properties (Galati and O'Brien 2004; Lee-Hilz *et al.* 2006). Furthermore, there are more and more indications that flavonoids are important signalling molecules, either regulating enzyme activity per se, or being modulators of cell signalling pathways and gene transcription of specific enzymes (Williams *et al.* 2004).

Much interest exists in the chemopreventive activity of flavonoids. Because of their ability to modify expression levels of enzymes that detoxify carcinogens, flavonoid phytonutrients seem to be dietary compounds that effectively contribute to cancer prevention. The large variety of enzyme regulation and gene induction mechanisms by flavonoids will be presented in more detail in the following paragraphs.

2. Flavonoids as regulators of enzyme activity

Many flavonoids appear to have effects on the activity and expression levels of various enzymes. Flavonoids can modulate the activity and/or expression level of many enzyme classes. Yet, flavonoids do not cause widespread disturbances in metabolism. They can inhibit enzymes such as hyaluronidase (Li *et al.* 1997), phospholipase (Kyo *et al.* 1998), phosphatase (Na *et al.* 2006), cAMP-phosphodiesterase (Ruckstuhl and Landry 1981), quinone reductase (Varma *et al.* 1975; Lee *et al.* 2005), protein phosphokinase (Jinsart *et al.* 1992), lipoxygenase (Leung *et al.* 2007), cyclooxygenase (Guerra *et al.* 2006), nitric oxide synthase (Guerra *et al.* 2006), tyrosinase (Kim *et al.* 2006), thyroid peroxidase (Doerge and Chang 2002), xanthine oxidase (Dew *et al.* 2005), glutathione S-transferase (van Zanden *et al.* 2003) and succinate dehydrogenase (Hodnick *et al.* 1994). This list of enzymes inhibited by flavonoids is far from complete and frequently new reports appear.

The ability of flavonoids to regulate enzyme activity contributes to their reported bioactivity. The anti-viral activity, for example, has been attributed to the efficiencies of flavonoids to inhibit HIV1-protease and reverse transcriptase (Spedding *et al.* 1989; Xu *et al.* 2000). Flavonoids are also potent inhibitors of mitogen signalling processes by affecting various kinase activities (Reiners *et al.* 1998). Furthermore, flavonoids, such as quercetin, myricetin and morin, exhibit anti-tumor activity by inhibiting topoisomerase I and II (Constantinou *et al.* 1995). However, the inhibition of topoisomerase II has also been linked with infant leukaemia (Spector *et al.* 2005). This contradiction reveals the complexity of the bioactivity spectrum of flavonoids.

In some cases, the type of inhibition is competitive, but more often it is allosteric. Therefore, the ability of flavonoids to act as regulators of enzyme activity is highly dependent on the individual structure. For example, flavonoids can inhibit or stimulate enzymatic activity of human cytochromes P450 depending upon their chemical structure. Protein-flavonoid complex formation depends both on the three dimensional structure of flavonoids and their electrostatic properties. Areas of highest electronegativity occur at the 5-hydroxyl and 7-hydroxyl positions of the A-ring, at the various hydroxyl groups of the B-ring, and at the 3-hydroxyl position of the C-ring (Fig. 1.1). The hydroxyl moieties are potential sites for hydrogen bonding with protein residues, for interaction with metal cations, and for electron transfer. Flavonoids possessing hydroxyl groups inhibit CYP-dependent monooxygenase activity, whereas those lacking hydroxyl groups can stimulate the enzyme activity (Hodek *et al.* 2002). SIRT1 (Silent Information Regulator Two ortholog 1), the human ortholog of the yeast sir2

protein, an important regulator of lifespan extension, is reported to be stimulated by myricetin but not by quercetin (de Boer *et al.* 2006b).

The next paragraph will focus in detail on the inhibitory effect of flavonoids on the important detoxification enzyme, NAD(P)H: quinone oxidoreductase 1.

2.1 NQO1

In vitro inhibition studies have shown that flavonoids are able to inhibit the detoxification enzyme NAD(P)H: quinone oxidoreductase 1 (NQO1; EC 1.6.99.2), formerly referred to as DT-diaphorase (Chen *et al.* 1993). NQO1 is a homodimeric, cytosolic flavoprotein with each subunit containing 273 amino acid residues. The crystal structure of NQO1 is shown in Figure 1.4.

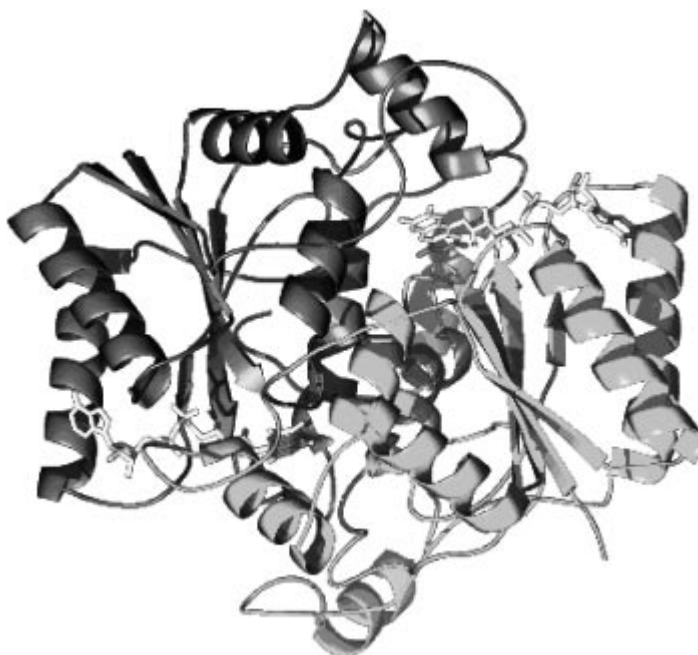


Figure 1.4: Crystal structure of NQO1 (PDB code 1DXO) (Faig *et al.* 2000)

NQO1 can catalyse the two-electron reduction of quinones to hydroquinones, thereby preventing the one electron reduction of quinones by cytochromes P450 and their redox cycling with molecular oxygen generating superoxide radicals, as well as their electrophilic activity resulting in covalent interaction with cellular macromolecules (Ross and Siegel 2004). NQO1 functions via 'ping-pong' kinetics involving two half-reactions (Li *et al.* 1995). In the reductive half-reaction NAD(P)H reduces the FAD co-factor and NAD(P) is released. In the oxidative half reaction, the quinone substrate reoxidizes the flavin and the hydroquinone product is released. NQO1 displays

activity towards a wide variety of substrates including quinones, quinone-imines, glutathionyl-substituted naphthoquinones, dichlorophenolindolphenol, methylene blue, and also azo and nitro compounds (Ross and Siegel 2004). It also participates in reduction of endogenous quinones, such as vitamin E quinone and ubiquinone, generating antioxidant forms of these molecules (Beyer *et al.* 1996; Siegel *et al.* 1997). NQO1 has recently been shown to interact with superoxide and may be involved in scavenging superoxide within the cell (Siegel *et al.* 2004). Another important property of NQO1 is its ability to stabilize the p53 tumor suppressor protein and consequently contributes to p53-dependent stress responses (Gong *et al.* 2007). With regard to the functional genetics of NQO1, the homozygous C609T mutation (187Pro → Ser) is associated with lack of activity. NQO1 609CC has high activity, whereas 609CT has low to intermediate activity and 609TT has no activity. The homozygous mutation rates among Caucasian/African-American, Hispanic, and Asian populations are 5%, 15%, and 20%, respectively (Smith *et al.* 2005). Several studies have shown that the NQO1 C609T polymorphism is positively associated with childhood leukaemia (Krajinovic *et al.* 2002; Smith *et al.* 2002) and other types of blood dyscrasia in workers exposed to benzene (Iskander and Jaiswal 2005).

Several inhibitors of NQO1 are known and some examples are depicted in Figure 1.5.

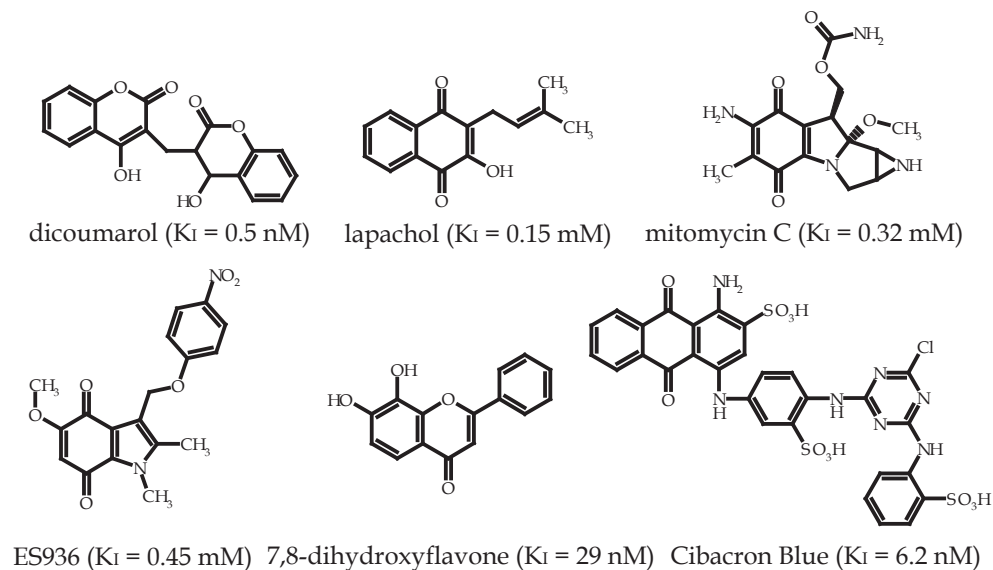


Figure 1.5: Reported inhibitors of NQO1 activity (Preusch 1986; Schlager and Powis 1988; Presteria *et al.* 1992; Chen *et al.* 1993; Winski *et al.* 2001)

Dicoumarol is the most potent inhibitor of NQO1 activity, known so far (Ernster *et al.* 1960). The inhibition of NQO1 by dicoumarol is competitive with respect to NAD(P)H. Recently, Asher and coworkers (Asher *et al.* 2006) obtained a crystal structure of NQO1 in complex with dicoumarol, revealing that residues Tyr128 and Phe232, located in the catalytic pocket of NQO1, are involved in the conformational changes upon dicoumarol binding.

Flavonoids were also identified as NQO1 inhibitors, strongly inhibiting NQO1 activity, competitive with respect to NAD(P)H (Liu *et al.* 1990; Chen *et al.* 1993). *In vitro* inhibition studies revealed inhibition constants of 29 nM and 30 nM for 7,8-dihydroxyflavone and 5,7-dihydroxyflavone, respectively (Chen *et al.* 1993). Preliminary molecular dynamics studies suggested that these compounds bind differently in the active site of NQO1 than dicoumarol and that the 7-hydroxyl moiety of the flavonoids interacts with His161 in the active site (Chen *et al.* 1999). The inhibition of NQO1 by flavonoids is pointing at a mechanism contradicting the proven beneficial properties of these phytochemicals.

3. Flavonoids as regulators of gene expression

Dietary flavonoids are regulators of gene expression and can exert their effects on various pathways separately or sequentially. By modulating cell signaling pathways, flavonoids for example can activate cell death signals and induce apoptosis in precancerous or malignant cells resulting in the inhibition of cancer development or progression (Brusselmans *et al.* 2005). However, this regulation of cell signalling by flavonoids can also lead to cell proliferation/survival or inflammatory responses due to increased expression of several genes (van der Woude *et al.* 2003; Kumazawa *et al.* 2006). The occurrence of crosstalk between pathways, stimulated or inhibited by flavonoids is complex and not very well understood. The action of flavonoids as regulators of gene expression includes their ability to exert phytoestrogen-like activity, inducing the estrogen receptor (ER)-mediated gene expression (van der Woude *et al.* 2005b). Flavonoids can also mediate apoptosis in HOS cells, driven by induction of p53-mediated mitochondrial stress and nuclear translocation of the apoptosis inducing factor (AIF) and endonuclease G (EndoG) (Kook *et al.* 2007). Furthermore, flavonoids can affect the cellular signalling pathways that regulate proliferation, survival and transformation of cells, important for cancer biology. They have been reported to regulate phosphoinositide 3-kinase (PI3-kinase), Akt/protein kinase B (Akt/PKB), tyrosine kinases, protein kinase C (PKC), and mitogen activated protein kinase (MAPK) signalling cascades (Williams *et al.* 2004). Flavonoids can modulate the MAPKs activities (ERK2, JNK1, and/or p38), which in turn regulate gene expression (Kuo and Yang 1995). Flavonoids were also reported to act as natural ligands for the aryl hydrocarbon receptor (AhR) (Ashida *et al.* 2000; Tutel'yan *et al.* 2003), which is a ligand-dependent transcription factor for the xenobiotic-responsive element (XRE), a specific DNA sequence in the enhancer regions of phase 1 enzymes, like CYP1A1 involved in xenobiotic metabolism (Denison *et al.* 1988). Another important signalling

pathway affected by flavonoids is the activation of redox-sensitive transcription factors such as Nrf2, p53, AP-1, NF- κ B (Fresco *et al.* 2006). Loss of antioxidants and/or increases in ROS is detrimental to normal cellular function as this situation can result in lipid peroxidation, enzyme inactivation and DNA damage. The cell has therefore evolved processes that allow it to survive exposure to agents that perturb the redox balance whilst maintaining its genetic stability through an adaptive program of gene expression. All these transcription factors transduce changes in the cellular redox-status and modulate gene expression responses to oxidative and electrophilic stresses, presumably via sulfhydryl modification of critical cysteine residues found on these proteins and/or other upstream redox-sensitive molecular targets (Hansen *et al.* 2006). The following paragraphs will focus in more detail on the redox-sensitive transcription factor Nrf2 and its regulation of EpRE-mediated gene expression and on the XRE, which are both important regulatory elements in the NQO1 regulatory region.

3.1 Electrophile-responsive element

Cells can respond to dietary phytochemicals, such as flavonoids, through a receptor protein-based sensing mechanism leading to altered gene expression, resulting in pharmacologically beneficial effects. The transcription of several classes of genes that respond to oxidative stress and act in concert to protect against acute chemical insult, for example by elevating cellular antioxidant levels, removing damaged macromolecules and repairing DNA, is of great importance for the cellular survival. Especially the transcription of the *NQO1* gene, induced by electrophilic and/or oxidative insult, is of particular interest with regards to chemoprevention of cancer (Prochaska and Santamaria 1988; Wang and Williamson 1996; Nioi and Hayes 2004). Various flavonoids were reported to induce the gene expression of NQO1 (Yannai *et al.* 1998). The transcriptional regulation of NQO1 protein production occurs through the electrophile-responsive element (EpRE) (Chen and Kong 2004) initially referred to as the antioxidant-responsive element (ARE) (Prester and Talalay 1995). The EpRE transcriptional enhancer element is located in the 5'-flanking region of the respective genes and is involved in the coordinated transcriptional activation of genes involved in protection against oxidative stress and other cancer-chemoprotective mechanisms. The key regulator of EpRE-mediated gene expression is the transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2) which is a member of the nuclear basic leucine zipper transcription factors (Jaiswal 2004). Nrf2 is an unstable protein, which maintains a low basal level of cytoprotective gene expression under homeostatic conditions (Itoh *et al.* 2003). Keap1 (Kelch-like erythroid cell-derived protein with CNC homology-associating protein 1) is a substrate adaptor, which regulates the cellular level of Nrf2 by bridging Nrf2 with Cul3-BTB^{Keap1}E3 ligase (Kobayashi *et al.* 2004; Tong *et al.* 2006b) leading to its ubiquitination and degradation.

Keap1 is a thiol-rich dimeric protein (Itoh *et al.* 1999), which comprises 5 domains; the N-terminal region (NTR), the BTB protein-protein interaction motif (BTB), the intervening region (IVR), the double glycine repeat (DGR) and the C terminal region (CTR) (Fig. 1.6).

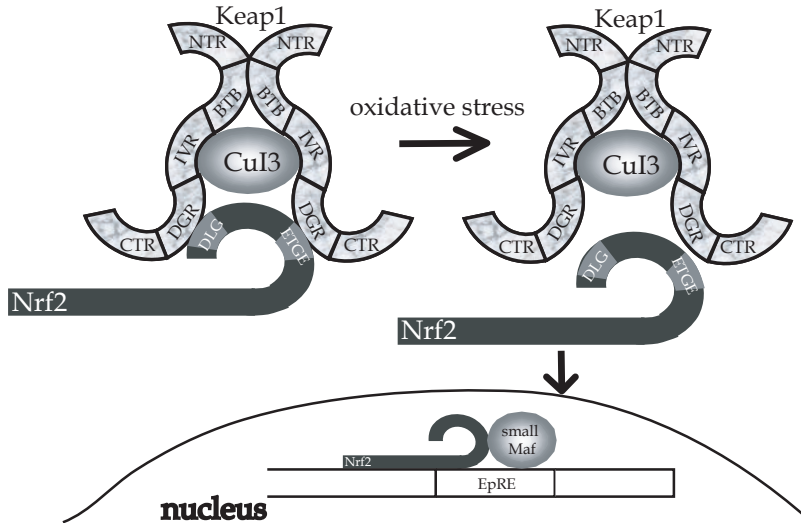


Figure 1.6: Mechanism of EpRE-mediated gene expression. Release of Nrf2 from Keap1 and nuclear translocation of Nrf2, resulting in EpRE-mediated gene expression

As a protective response to electrophilic chemicals and oxidative stress, Nrf2 is able to escape Keap1-mediated repression, translocates to the nucleus and activates the expression of its target genes by binding to the EpRE site as a heterodimer with other positively or negatively regulating transcription factors, such as small Maf protein (Fig. 1.6) (Kwong *et al.* 1999; Katsuoka *et al.* 2005).

The fact that Keap1 contains multiple cysteines, many of which are potential sites of oxidative attack by inducers of the EpRE-mediated gene expression, has led to the suggestion that the Keap1-Nrf2 interaction constitutes a sensor of oxidative stress involved in triggering EpRE-controlled responses to restore the physiological redox status in cells (Zhang and Hannink 2003; Kobayashi *et al.* 2004; Dinkova-Kostova *et al.* 2005b). The release of Nrf2 from Keap1 is suggested to be the result of an interaction of inducers with reactive thiol groups of the Keap1 protomers, which are holding Nrf2 in complex, resulting in disulfide formation and conformational changes of Keap1 (Dinkova-Kostova *et al.* 2005a; Dinkova-Kostova *et al.* 2005b).

Another proposed mechanism of increased EpRE-mediated gene induction by nuclear Nrf2 accumulation is through more indirect mechanisms, including phosphorylation of Nrf2 by protein kinase C (PKC) at Ser40 leading to the dissociation of Nrf2 from

the complex (Huang *et al.* 2002; Bloom and Jaiswal 2003; Numazawa *et al.* 2003). Phosphorylation of Nrf2 leads to an increase in its stability and subsequent transactivation activity (Nguyen *et al.* 2003). Furthermore, PKC plays a role in formation of peroxynitrite that activates Nrf2 for glutathione S-transferase induction and PKC serves as activator downstream of PI3-kinase (Kim and Kim 2004).

3.2 Xenobiotic-responsive element

Flavonoids can exert protective effects by selectively inhibiting or stimulating key proteins in cell signalling cascades and modulate the activity of enzymes, including phase 1 and phase 2 biotransformation enzymes. Although flavonoids have attracted considerable attention as inducer of enzymes involved in phase 2 biotransformation mediated by the EpRE (Valerio *et al.* 2001), several flavonoids are also reported to induce enzymes involved in phase 1 biotransformation, such as CYP1A1 (Canivenc Lavier *et al.* 1996; Ciolino *et al.* 1999). Flavonoids can act as natural ligands for the AhR receptor (Ashida *et al.* 2000; Tutel'yan *et al.* 2003). Beside phase 1 enzymes, AhR also regulates the transcription of NQO1 and certain glutathione S transferases, which have, beside an EpRE, a functional XRE in their enhancer region (Jaiswal 1991; Nebert and Duffy 1997; Ma *et al.* 2004). It is proposed that EpRE-mediated gene expression is partly regulated by AhR inducers by activating the XRE (Miao *et al.* 2004). This suggests a direct relation between AhR and EpRE and places the EpRE-pathway downstream of XRE-mediated gene expression (Miao *et al.* 2005). Thus, cross-talk between the AhR and the Nrf2, and a functional interaction between the AhR/XRE and the Nrf2/EpRE pathways might be required for the transcription of the NQO1 gene (Ma *et al.* 2004).

4. Aim and outline of this research

The protective effect of phytochemicals, such as flavonoids can be caused by modulating the activity of enzyme systems responsible for deactivation of chemical carcinogens, such as NQO1. Several flavonoids act as NQO1 inducers by activating NQO1 gene transcription through the electrophile-responsive-element (EpRE) to increase NQO1 mRNA levels. On the other hand, certain flavonoids are efficient inhibitors of the NQO1 enzyme activity *in vitro*. The objective of this project is to elucidate the complex interplay between flavonoids and NQO1 at the protein and at the gene level.

The first part of the project deals with the inhibitory effect of flavonoids at the NQO1 protein level. For this, NQO1 was purified and the inhibitory effect of several flavonoids on the enzyme activity was tested. Furthermore, molecular dynamics studies were performed to get a deeper insight in the NQO1-flavonoid interaction underlying the inhibition (**Chapter 2**). After studying the *in vitro* inhibition of NQO1 by flavonoids, we developed a method to monitor NQO1 activity in living Chinese hamster ovary (CHO) cells genetically engineered to overexpress human NQO1. This method allowed us to determine NQO1 inhibition by flavonoids in cells without disruption

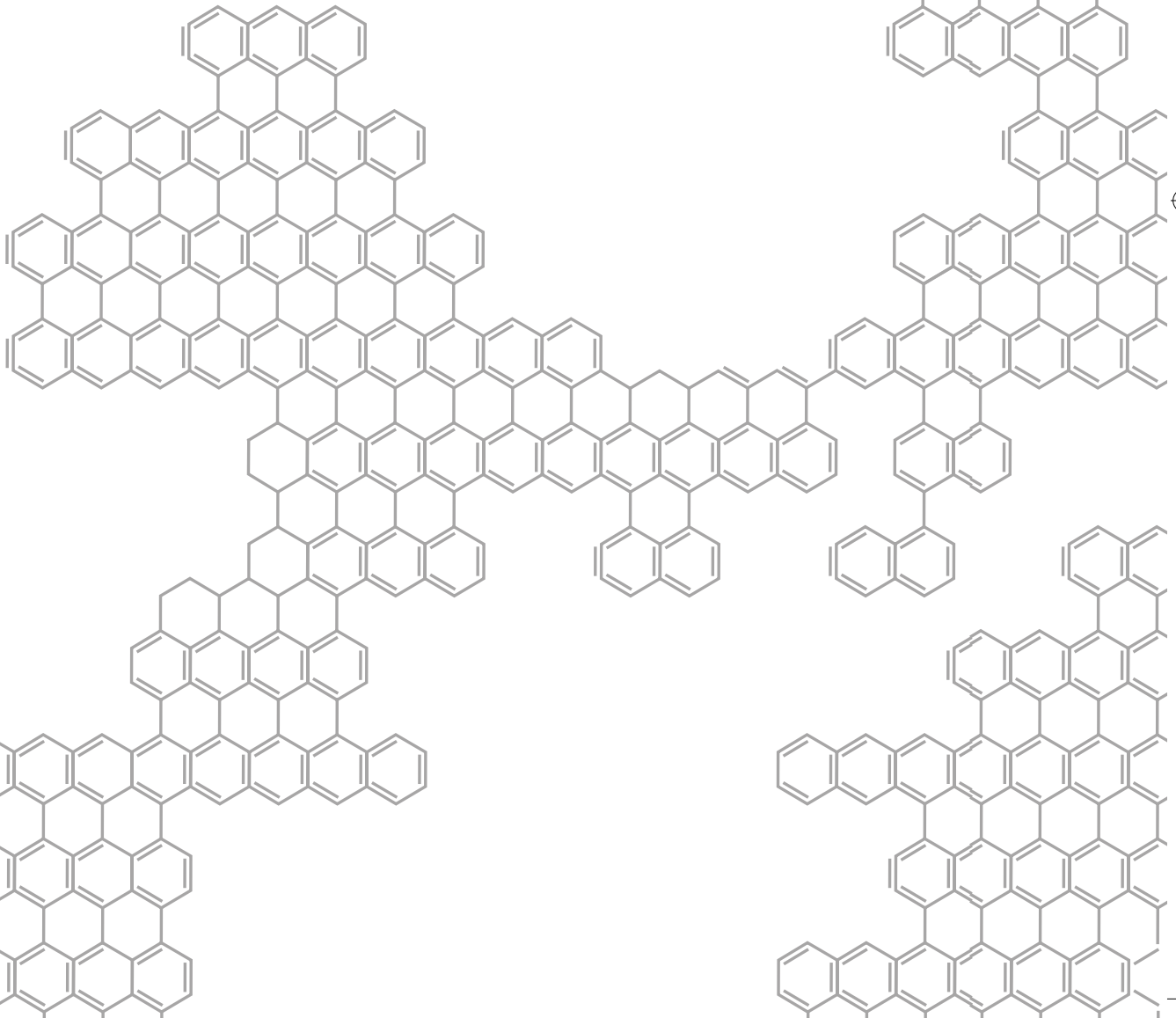


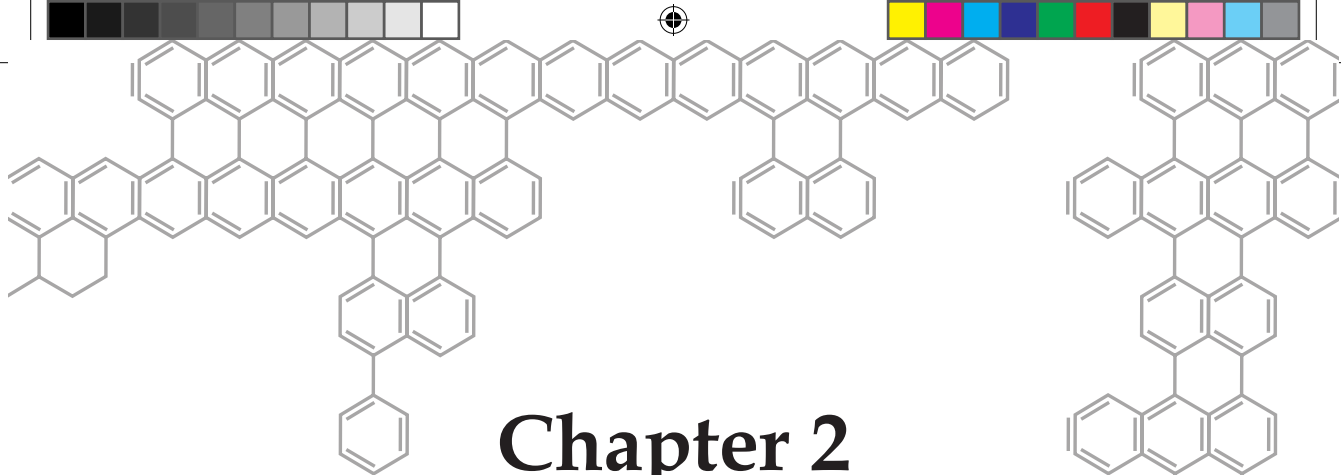
Chapter 1

of the cells or addition of cofactors, enabling the assessment of enzymatic activity and the interaction of modulators of enzymatic activity in an intracellular situation (**Chapter 3**).

The second part of this project focused on the induction of the *NQO1* gene by flavonoids. These bioactive food components are able to induce XRE- and EpRE-mediated gene expression, resulting in the induction of phase 1 and phase 2 biotransformation enzymes. Beside the EpRE enhancer element, the *NQO1* gene has a functional XRE in its regulatory region. To test whether the flavonoid-induced EpRE-mediated gene expression is a result of upstream XRE-mediated gene expression, flavonoids were tested for their ability to induce XRE- and EpRE-mediated gene expression by use of two reporter genes cell lines, consisting of Hepa-1c1c7 cells stably transfected with a firefly luciferase reporter gene under expression regulation of an EpRE- or an XRE-containing sequence (**Chapter 4**). **Chapter 5** and **6** present studies on the mechanism by which flavonoids are able to induce the EpRE-mediated gene expression, for example of the *NQO1* gene or of other detoxification enzymes genes. The role of PKC in the flavonoid-induced activation of EpRE-mediated gene expression is studied and the flavonoid-induced release of Nrf2 from the Keap1 complex is further elucidated. Due to the extensive metabolism of flavonoids to glucuronides *in vivo*, questions were raised if studies conducted with quercetin aglycone, stating its health-promoting activity, are of actual relevance. Therefore, **Chapter 7** presents results of our study investigating the ability of quercetin phase II metabolites to induce EpRE-mediated gene expression. Finally, the results and findings are summarized in **Chapter 8**.



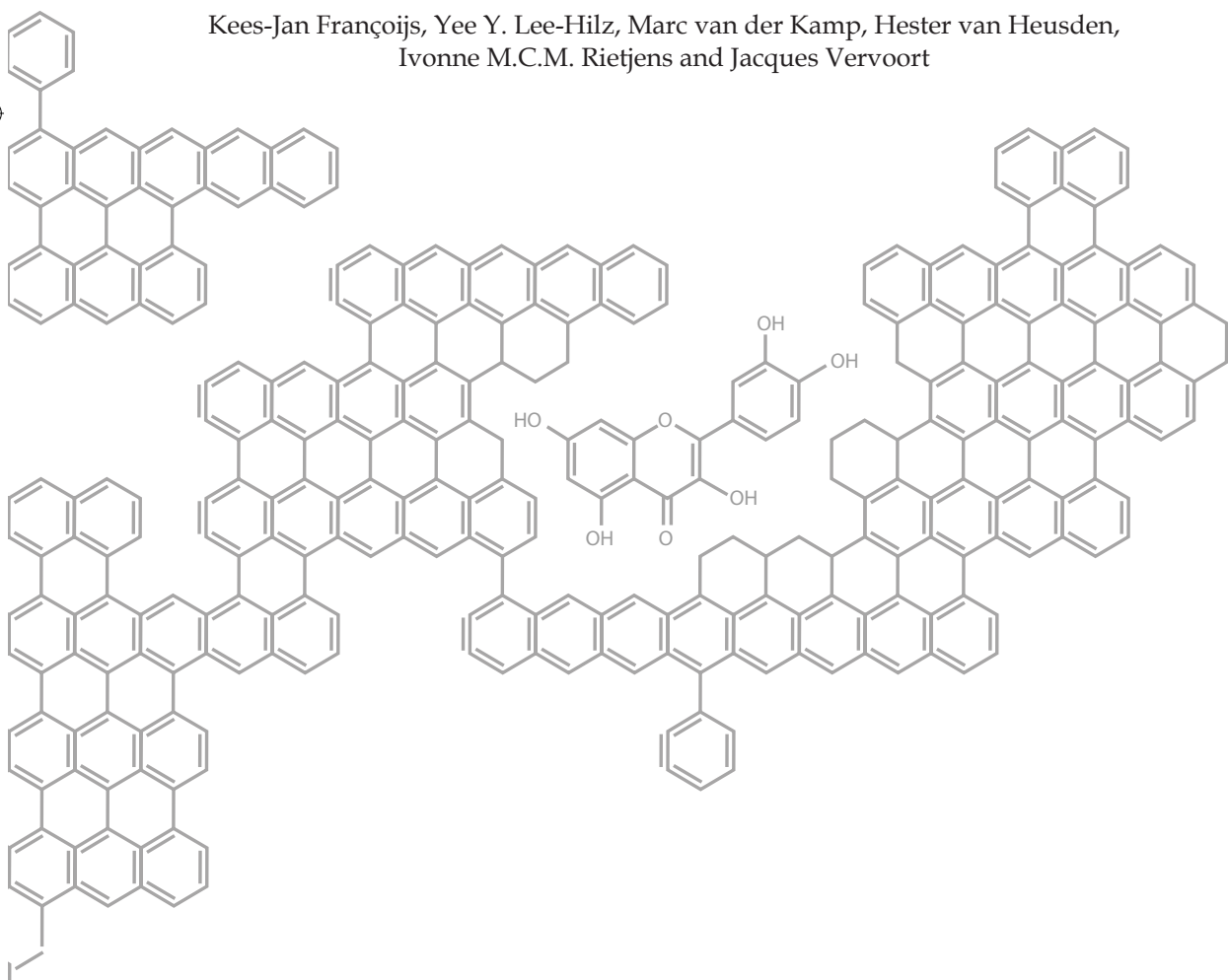




Chapter 2

A structural model for the inhibition of NAD(P)H: quinone oxidoreductase 1 by flavonoids

Kees-Jan Françoijis, Yee Y. Lee-Hilz, Marc van der Kamp, Hester van Heusden,
Ivonne M.C.M. Rietjens and Jacques Vervoort



Abstract

NAD(P)H: quinone oxidoreductase 1 (NQO1) is a multi-tasking enzyme, involved in the maintenance of the redox-balance in cells. This detoxification enzyme is inhibited by flavonoids, plant-derived phytochemicals, associated with reduced risk of many diet-related chronic diseases. Among various flavonoids tested, 7,8-dihydroxyflavone appeared to be the most efficient inhibitor of both the human and the rat enzyme. Inhibition and molecular docking studies were performed to describe a model for flavonoid inhibition of human NQO1. Using Autodock, the lowest energy docking modes for 7,8-dihydroxyflavone for the dimeric protein were selected. These lowest energy docking modes were followed by CHARMM optimization, which resulted in the description of a binding mode for 7,8-dihydroxyflavone. Based on the experimental and theoretical approaches in our study we show that 7,8-dihydroxyflavone is a competitive inhibitor towards NADH. The binding interaction of 7,8-dihydroxyflavone with human NQO1 is described in detail.

Introduction

NAD(P)H: quinone oxidoreductase 1 (NQO1, E.C. 1.6.99.2), also referred to as DT-diaphorase (Ernster and Navazio 1958), nicotineamide quinone oxidoreductase 1 or menadione reductase, is a homodimeric, ubiquitous, cytosolic flavoprotein (Ross and Siegel 2004). This enzyme has been described to be present in various species including mouse, rat and human (Jaiswal *et al.* 1988). NQO1 is involved in protection against toxic agents and reactive forms of oxygen by single-step detoxification of quinones, maintenance of endogenous antioxidants in their reduced form and regulation of p53 tumor suppressor protein (Ross 2004). The best-described function of NQO1 is its ability to perform two-electron reduction of quinones and nitro aromatics (Lind *et al.* 1982), thereby preventing the one electron reduction of quinones by cytochromes P450 and their redox cycling with molecular oxygen generating superoxide radicals (Dinkova-Kostova and Talalay 2000). The catalytic activity of NQO1 functions via 'ping-pong' kinetics, whereby NAD(P)H binds to the active site, reduces the flavin co-factor to FADH₂, and is then released prior to substrate binding and complete reduction of the substrate by hydride transfer (Li *et al.* 1995). The molecular basis of this 'ping-pong' mechanism is provided by the significant overlap between the NAD(P)H and the quinone binding site (Li *et al.* 1995). NQO1 displays broad substrate specificity and its catalytic functionality is not restricted to quinones but also includes quinone-imines, nitro and azo compounds (Lind *et al.* 1982).

Several inhibitors of this detoxification enzyme are known. Dicoumarol and other anti-coagulants are reported to be competitive inhibitors of NQO1 activity (Ernster *et al.* 1960). Furthermore, flavonoids, polyphenols naturally occurring in fruits and vegetables, which are contributing to the beneficial health effects of fruits and vegetables consumption, were also identified as NQO1 inhibitors, strongly inhibiting NQO1 activity *in vitro* (Liu *et al.* 1990; Chen *et al.* 1993; Lee *et al.* 2005). In the past, several studies were done on the kinetic characteristics of NQO1 inhibition, but the results reported so far are contradictory. Although the inhibition of NQO1 by dicoumarol, phenindone or flavonoids are reported to show competitive behavior with respect to NAD(P)H, some kinetic studies claimed that these compounds bind at a site different from the NAD(P)H binding site (Liu *et al.* 1990; Chen *et al.* 1993). Recently, Asher and coworkers (Asher *et al.* 2006) obtained a crystal structure of NQO1 in complex with dicoumarol, revealing that Tyr128 and Phe232, which are present in the catalytic pocket of NQO1, are involved in the conformational changes upon dicoumarol binding. However, enzyme proteolysis experiments and kinetic studies upon concomitant addition of two inhibitors (Yonetani and Theorell 1964) suggested that the flavonoids do not bind at the same site as dicoumarol (Chen *et al.* 1993). Some initial docking data of chrysin binding to the active site of NQO1 showed, that the C-8 carbon of chrysin is close to Gly 150 and the 4-keto group is pointing away from Gly 150 towards the solvent (Chen *et al.* 1999). Inhibition studies of NQO1 by irreversible binding of 5-hydroxy-7-bromoacetylflavone indicate that a histidine residue is present at the flavonoid-binding site (Chen *et al.* 1995).

However, so far the specific binding mechanism of flavonoids to NQO1 is not known.

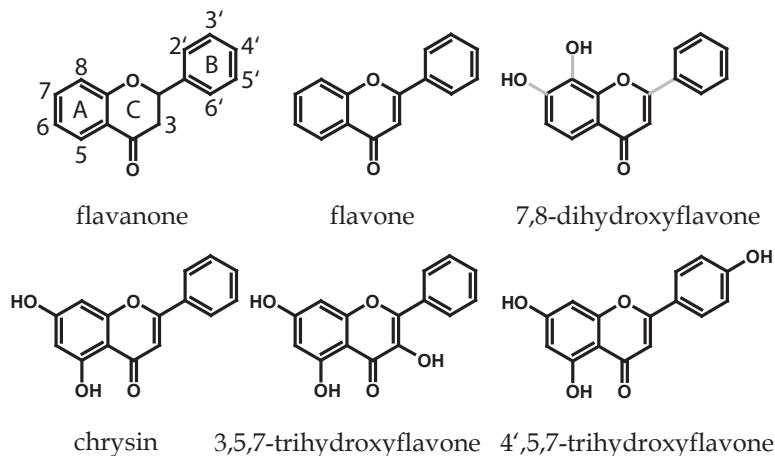


Figure 2.1: Structural representation of the flavonoid inhibitors used in the present study and of chrysin, the flavonoid-type NQO1 inhibitor studied by Chen and coworkers (Chen *et al.* 1999). Rotatable bonds of 7,8-dihydroxyflavone are highlighted in grey.

The objective of the present study is to obtain a more detailed model for the binding of flavonoids to NQO1. Therefore, a molecular modeling docking study was performed and combined with the experimental data of NQO1 inhibition by flavonoids. As model compound for the docking study, 7,8-dihydroxyflavone, representing a strong NQO1 inhibitor, was chosen. From the results obtained, we propose that the residues His 161, Tyr 128 and Try 126 in the active side of NQO1 are important for 7,8-dihydroxyflavone binding to NQO1.

Materials and Methods

Chemicals

Dicoumarol, CibacronBlue3G-A-agarose type-3000-CL, 3-[4,5-dimethylthiazo-2-yl]2,5-diphenyltetrazoliumbromide (MTT) and isopropyl- β -D-thiogalactopyranoside (IPTG) were purchased from Sigma (St. Louis, USA) and 1,4-naphthoquinone (menadione) was purchased from Fluka Chemika (Neu-Ulm, Switzerland). Dimethyl sulfoxide (DMSO) was obtained from Acros Organics (New Jersey, USA). 3,5,7-Trihydroxyflavone (galagin), 4',5,7-trihydroxyflavone (naringenin), 7,8-dihydroxyflavone, flavanone and flavone were purchased from Extrasynthese (Genay Cedex, France). NADH was obtained from Roche (Mannheim, Germany). For all standards and substrates a fresh stock solution in DMSO was prepared for each experiment.

Enzyme purification

Human NQO1 was overexpressed in *E.coli*, according to the procedure of Ma and coworkers (Ma *et al.* 1990). Briefly, *E.coli* BL21 (DE3) strain, transformed with the pT7-7DTD construct was inoculated in TY-Amp solution. Cells were grown (200 rpm, 30°C) until the optical density at 600 nm reached 0.5. NQO1 production is induced by IPTG and cells were harvested after 9 hours by centrifugation and French press techniques. The NQO1 enzyme from rat was obtained from rat liver cytosol. Purification was carried out using the procedure described by Prochaska and Talalay (Prochaska and Talalay 1988) with protamine sulphate precipitation, followed by cibacron blue chromatography, carried out at 4°C. For stability reason, the purified protein was stored at 4°C.

Enzyme activity

The human and rat NQO1 NADH-menadione reductase activity was determined spectrophotometrically as described earlier (Chen *et al.* 1993). Briefly, a catalytic amount of NQO1 was incubated with 1 mL of assay mixture containing 50 mM potassium phosphate pH 7.5, 200 μ M NADH, 160 μ M menadione, and 0.3 mg/mL of MTT. Menadione is the electron acceptor, and MTT is included for continuous re-oxidation of the menadiol formed. The reduction of MTT defines the NQO1 activity and can be measured at 610 nm ($\epsilon_{\text{MTT}} = 11.3 \text{ mM}^{-1}\text{cm}^{-1}$).

Docking of 7,8-dihydroxyflavone to NQO1

7,8-Dihydroxyflavone pretreatment for docking

7,8-Dihydroxyflavone was optimized using the Hartree-Fock method with the 6-31G* basis set in Gaussian03. Multi-orientation RESP fitting of 6 different orientations was performed using RED v1.0 to calculate the partial atomic charges. Subsequently, non-polar hydrogens were deleted and their charges were added to the corresponding heavy atoms. Bonds to the hydroxyl groups and the phenyl ring (ring B) were defined as rotatable bonds for flexible ligand docking (Fig. 2.1).

NQO1 pretreatment for docking

Protein and FAD coordinates were taken from chain A and C (a physiological dimer) from the X-ray crystal structure of human NQO1 co-crystallized with duroquinone (PDB-code 1DXO) (Faig *et al.* 2000). All crystallographic water molecules (and duroquinone) were discarded. The most probable tautomeric states of His residues were determined, which resulted in sensible hydrogen bonding networks. Hydrogen atoms were added to the structure and subsequently minimized by performing 3000 steps of conjugate gradient minimization in NAMD v2.5 (Kale *et al.* 1999), applying strict harmonic restraints (10 kcal/(mol*Å²)) on all heavy atoms. The AMBER 2003 force

field (Duan *et al.* 2003) was used for all protein atoms, and FAD parameters (oxidized form) were taken from AMBER Parameter Database (<http://pharmacy.man.ac.uk/amber/>). After minimization, only polar hydrogens were kept for docking.

Docking 7,8-dihydroxyflavone to NQO1

A 70x70x70 Å grid covering the complete dimer, with 0.556 Å grid spacing was calculated (centered on the geometric center of the dimer). The Lamarckian genetic algorithm (LGA) implemented in AutoDock v3.0, was used to perform docking in this grid. 100 Independent simulations with populations consisting of 50 random structures were performed. The maximum number of energy evaluations was 250,000, resulting in an average of 144 generations per simulation. The best individual of each generation survived. The probability for performing a local search of up to 300 pseudo Solis&Wets optimization iterations was 6%. Resulting structures were clustered with an RMS-tolerance of 2.5 Å.

Next, two 20x20x20 Å grids with 0.16 grid spacing (centered on both the C1 positions of duroquinone near the FAD of chain A and C of the original crystal structure) were calculated. 50 Independent simulations were performed in both grids using the Lamarckian genetic algorithm with parameters as specified above. Clustering of structures resulting from these docking runs was performed with an RMS-tolerance of 1.0 Å. Docking was performed on the complete NQO1 dimer and on the two active sites.

Molecular dynamics simulations

General amber force field (GAFF) atom types were assigned to 7,8-dihydroxyflavone using the Antechamber suite (Wang *et al.* 2004). Partial charges were obtained with RED v1.0, as described above. Protein and FAD parameters were as specified above. For the five different binding modes with the lowest energy, obtained from the docking of 7,8-dihydroxyflavone to both active sites of NQO1, minimization and MD-simulation was performed. The NQO1 dimer and ligand of each binding mode was solvated in a box of TIP3P water (Jorgensen *et al.* 1983), extending at least 10 Å from any solute atom. Counter-ions (Cl⁻) were added to neutralize the system

Conjugate gradient minimization and MD simulation was performed using NAMD v2.5 (Kale *et al.* 1999). For both minimization and MD simulation, periodic boundary conditions were used. Electrostatic interactions were calculated using the Particle-Mesh-Ewald full electrostatics method as implemented in NAMD, with a grid density of at least 1 Å⁻¹. Van der Waals interactions were smoothly truncated at 10 Å by applying a switch function from 8 Å to 10 Å. For MD simulation, an integration time step of 2.0 fs was used, applying SHAKE (Ryckaert *et al.* 1977) to constrain all bonds involving hydrogens.

To equilibrate the solvent, a short MD-protocol was used, keeping all solute heavy atoms fixed. First, solvent and hydrogen positions were relaxed by 3000 steps conjugate gradient minimization. Then, solvent and hydrogens were gradually heated during 4 ps of MD from 50K to 300 K, in steps of 50 K, using temperature reassignment every 0.5 ps. Then, a 16 ps MD simulation was performed in the NVT ensemble, using Langevin dynamics for temperature control, with a Langevin damping coefficient of 5/ps.

The interaction energy between 7,8-dihydroxyflavone and the complete dimer (for the five different binding modes, obtained from docking of 7,8-dihydroxyflavone to the two active sites of NQO1) was calculated after the first minimization of the total system, and after the minimization of the system following the MD simulation. Interaction energy was also calculated every 2 ps during the 100 ps production simulation and these energies were averaged. For interaction energy calculation, non-bonded interactions were smoothly truncated at 12 Å by applying a switch function from 10 Å to 12 Å.

Results

Inhibition of rat and human NQO1 by flavonoids.

Flavone, flavanone, 7,8-dihydroxyflavone and 3,5,7-trihydroxyflavone and 4',5,7-tri-hydroxy-flavanone were chosen as model compounds to test their ability to inhibit purified rat NQO1. From all the flavones tested only 7,8-dihydroxyflavone shows strong inhibition. Subsequently, the inhibition of human NQO1 by flavone and 7,8-dihydroxyflavone was tested. Both human and rat NQO1 show very similar inhibition profiles for flavone and 7,8-dihydroxyflavone (Fig. 2.2).

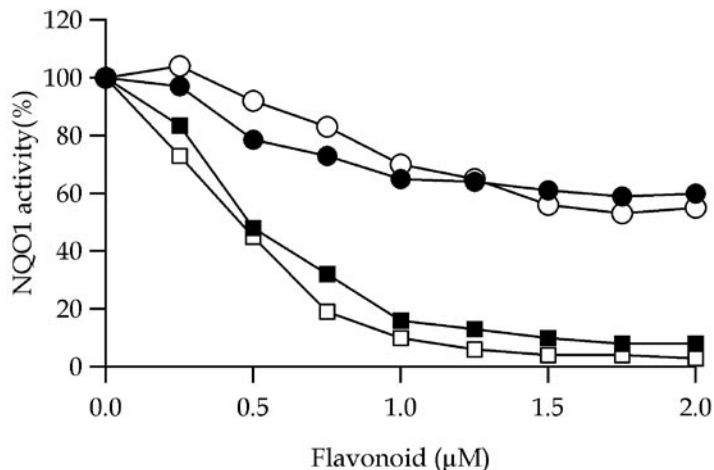


Figure 2.2: Concentration dependent inhibition of rat (white) and human (black) NQO1 by flavone (-○- and -●-) and 7,8-dihydroxyflavone (-□- and -■-).

In line with previous observations (Chen *et al.* 1993; Lee *et al.* 2005) 7,8-dihydroxyflavone was shown to be a potent flavonoid inhibitor of NQO1 activity, with an IC_{50} value of 0.6 μ M. Kinetic studies were performed with 7,8-dihydroxyflavone and the mode of flavonoid inhibition was shown to be competitive towards NADH (Fig. 2.3). Based on these results molecular docking studies were performed using 7,8-dihydroxyflavone.

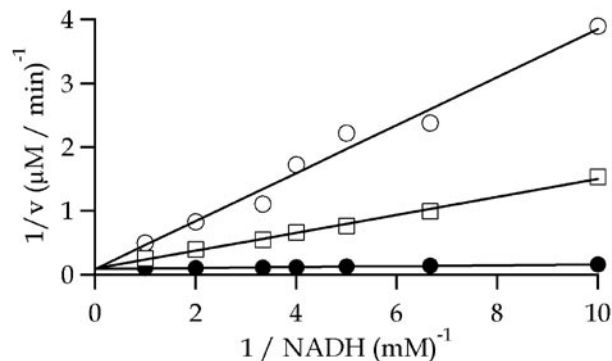


Figure 2.3: Inhibition of human NQO1 activity by 7,8-dihydroxyflavone.

Conditions: 5 ng/ml protein, 0.3 mg/mL MTT and varying concentrations of 7,8-dihydroxyflavone (-●- 0 μ M, -□- 0.8 μ M, -○- 1.2 μ M) and NADH. Data are presented as mean \pm standard deviation ($n = 4$).

Docking of flavonoids.

Docking of 7,8-dihydroxyflavone to the complete NQO1 dimer

LGA docking of 7,8-dihydroxyflavone was performed onto the human NQO1 dimer with heavy atom coordinates from the X-ray crystal structure (Pdb code 1DXO chain A and C) and 100 LGA runs were performed. After clustering with an RMS-tolerance of 2.5 \AA , 57 different clusters were found. Among those 57 clusters, 5 clusters have mean docked energies of at least 1 kcal/mol lower than the next best scoring cluster, representing the energetically most favorable binding sites. These 5 clusters were all located in the substrate or NAD(P)H nicotinamide binding region of one of the catalytic sites. All 5 clusters have binding orientations in which part of the 7,8-dihydroxyflavone is stacked onto the FAD isoalloxazine moiety. 2 Clusters with the lowest mean-docked energy are located in the active site near the FAD anchored in chain C of the original X-ray structure. The other 3 clusters with higher mean-docked energies are located in the active site near the FAD anchored in chain A (data not shown).

Docking of 7,8-dihydroxyflavone to the catalytic sites of the NQO1 dimer

More detailed LGA docking was performed in the catalytic site of each monomer. All docking results showed some kind of stacked position of 7,8-dihydroxyflavone onto the FAD isoalloxazine moiety. Docking in the active site at the C chain FAD (FAD anchored in the chain C) of the NQO1 crystal structure (1DXO) leads to energetically more favorable binding conformations than docking in the active site at the A chain FAD. The main difference between these two catalytic sites of the NQO1 dimer is the conformation of the Tyr128 side chain. This difference in orientation of the Tyr128 side chain is already present in the 1DXO X-ray 3D structure and influences the binding affinity of 7,8-dihydroxyflavone based on the docking results.

All docking results of the more detailed LGA docking could be grouped in 5 main binding modes. The interaction energies of the 5 binding modes, referred to as mode 1-5, are shown in Table 2.1.

Table 2.1: Results of the molecular dynamics run of the 5 different binding modes of 7,8-dihydroxyflavone in NQO1 compared to LGA docking data.

Docked energies and 7,8-dihydroxyflavone–NQO1 dimer force field interaction energies (E_I) are depicted at different stages of the minimization. For the average interaction energy during the 100 ps MD simulation, interaction energy was measured every 2 ps and subsequently averaged.

All energies are depicted in kcal/mole.

| binding mode | AutoDock docked energy | E_I after minimization | E_I after MD and minimization | average E_I over 100 ps MD |
|--------------|------------------------|--------------------------|---------------------------------|------------------------------|
| 1 | -11.05 | -16.42 | -17.38 | -12.09 |
| 2 | -10.31 | -7.34 | -9.55 | -3.12 |
| 3 | -10.09 | -17.00 | -17.16 | -25.85 |
| 4 | -9.58 | -39.03 | -22.16 | -26.19 |
| 5 | -9.14 | 21.54 | 45.60 | 20.26 |

Binding mode 1-4 are obtained from docking of 7,8-dihydroxyflavone to the active site of chain C (FAD anchored in chain C), whereas binding mode 5 was obtained from docking of 7,8-dihydroxyflavone to the active site of chain A (FAD anchored in chain A). These 5 binding modes are graphically shown in Fig. 2.4.

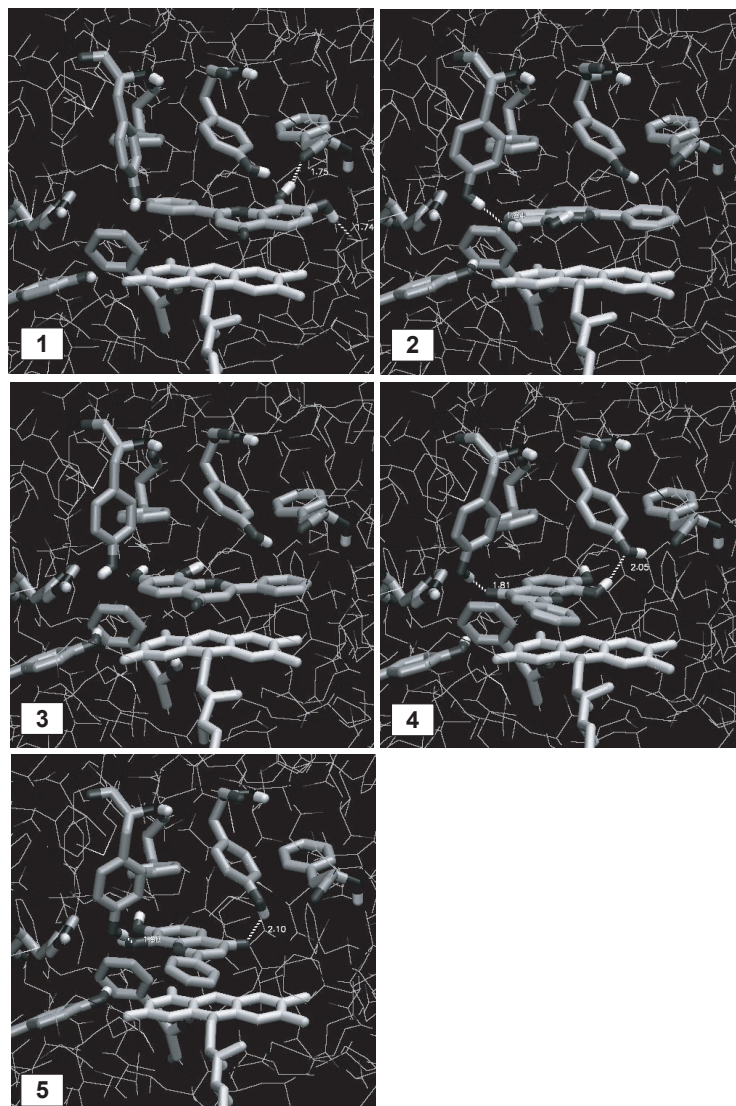


Figure 2.4: Five different binding modes of 7,8-dihydroxyflavone in the catalytic site of human NQO1 obtained with AutoDock3. FAD and important residues lining the active site are shown as sticks, as is 7,8-dihydroxyflavone itself. Hydrogen bonds between ligand and amino acid residues are indicated as dotted lines. Pictures were generated with VMD v1.8 (Humphrey *et al.* 1996).

The active sites of NQO1 are at the interface of the two chains. Therefore, for easier discrimination, the residues belonging to chain A will be marked with a hyphen. In binding mode 1, the A and C ring of 7,8-dihydroxyflavone are stacked on the

FAD benzene ring and the ketone oxygen is pointing out of the active site (Fig. 2.4.1). Hydrogen bond interaction is possible between the hydroxyl groups of 7,8-dihydroxyflavone and the backbone oxygens of Phe120' and Glu117'. In mode 2, the 7,8-dihydroxyflavone A ring is stacked on the FAD isoalloxazine pyrimidine ring and the hydroxyl groups of 7,8-dihydroxyflavone are directed outwards (Fig. 2.4.2). Hydrogen bond interaction is possible between the 7-OH group of 7,8-dihydroxyflavone and the Tyr128'-OH. Binding mode 3 can be characterized by a stacking interaction of the 7,8-dihydroxyflavone A and C rings onto the isoalloxazine pyrimidine and pyrazine rings, with the 7,8-dihydroxyflavone hydroxyl groups directed into the active site (Fig. 2.4.3). In binding mode 4, mainly the 7,8-dihydroxyflavone C ring is stacked on the FAD isoalloxazine pyrimidine ring, with the 7,8-dihydroxyflavone B-ring directed outwards (Fig. 2.4.4). Hydrogen bond interactions are possible between the carbonyl oxygen of 7,8-dihydroxyflavone and His161-NH and/or Tyr128'-OH and between the 7,8-dihydroxyflavone hydroxyl groups and Tyr126'-OH. Finally, binding mode 5 (Fig. 2.4.5), which was only found by docking in the catalytic site near FAD anchored in chain A, shows the 7,8-dihydroxyflavone C ring stacked onto the FAD isoalloxazine ring with the 7,8-dihydroxyflavone B ring directed outwards, like in binding mode 4. Compared to binding mode 4, mode 5 has the 7,8-dihydroxyflavone hydroxyl groups on the other side, towards His161' and Tyr128, with which hydrogen bond interactions are possible, as depicted in Fig. 2.4.5.

All 5 binding modes occupy a large part of the substrate and NAD(P)H nicotinamide binding region at the *si*-face of the isoalloxazine moiety of FAD, explaining the competition with the binding of NAD(P)H (Faig *et al.* 2000).

Molecular dynamics simulations of 5 binding modes of 7,8-dihydroxyflavone in the active site of human NQO1

To further assess the 5 possible binding modes obtained from docking in both catalytic sites of the human NQO1 dimer, minimization and MD-simulation was performed on these 5 binding modes. Figure 2.5 shows the 5 binding modes of NQO1 with 7,8-dihydroxyflavone at the catalytic site after minimization of the docked conformations.

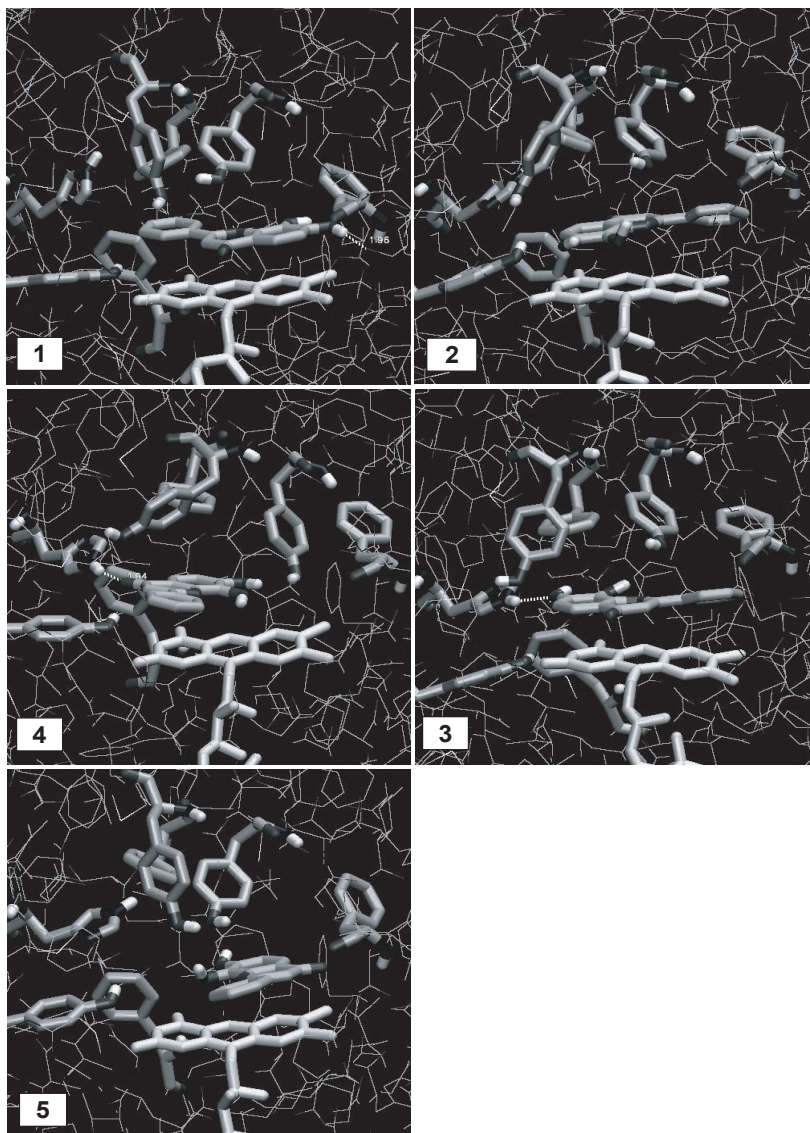


Figure 2.5: Five different binding modes of 7,8-dihydroxyflavone in the catalytic site of human NQO1, obtained after molecular dynamics simulation and conjugate gradient minimization. FAD and important residues lining the active site are shown as sticks, as is 7,8-dihydroxyflavone itself. Hydrogen bonds between ligand and amino acid residues are indicated as dotted lines. Pictures were generated with VMD v1.8 (Humphrey *et al.* 1996)

The molecular dynamics simulations, performed for all 5 binding modes in the NPT ensemble (constant pressure, 1 bar, and temperature, 300 K), resulted in slight changes in all binding modes leading to better stacking interaction of the flavone rings with the FAD isoalloxazine moiety and more favorable hydrogen bond interactions (Fig. 2.5). The distance between the plane of the C ring of 7,8-dihydroxyflavone and the plane of FAD was determined for all 5 binding modes and the average distance was 3.6 Å. Among the 5 binding modes, mode 4 seems to be most probable, due to its minimal changes after molecular dynamics simulations (Fig. 2.4.4 and 2.5.4). The position of 7,8-dihydroxyflavone and the position of the residues in the catalytic site of the NQO1 dimer do not change much during simulation. In this binding mode the keto group of 7,8-dihydroxyflavone can form a hydrogen bond with His 161 as well as with Tyr 128' (Fig. 2.5.4).

The results of the interaction energy analysis, shown in Table 2.1, confirms binding mode 4 to be most favorable. Binding mode 5 clearly is the least favorable after minimization, due to the fact that in the energy minimized conformation, there is no good stacking interaction and close contacts between several residue side-chains and 7,8-dihydroxyflavone (Fig. 2.5.5). Furthermore, for all binding modes the interaction energies were also calculated during a 100 ps MD simulation (every 2 ps) and for a conformation obtained by energy minimization after the MD simulation (Table 2.1). This analysis shows binding modes 3 and 4 to be the most energetically favorable. These modes are also relatively stable during the 100 ps MD simulation: no large changes in the conformation occur (Fig. 2.4, 2.5). However, binding mode 4 seems to be slightly better, especially when the interaction energies of mode 3 and 4 are compared after MD simulation and minimization (Table 2.1).

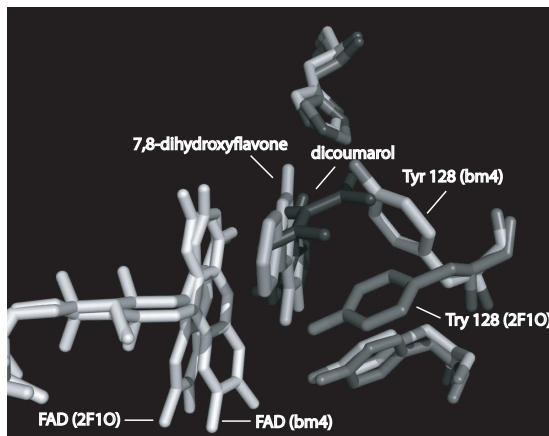
Discussion

NQO1 is an important cellular detoxification enzyme. *In vitro* studies have shown that the activity of NQO1 is inhibited by flavonoids, which would represent a mechanism opposing the reported beneficial effects of these phytochemicals. This paper presents a detailed model for the binding of 7,8-dihydroxyflavone to NQO1, elucidating its binding interactions. The experimental data of this study show that 7,8-dihydroxyflavone is the best NQO1 inhibitor among the flavonoids tested. This is in line with earlier studies in which also other flavonoids were included in the comparison (Chen *et al.* 1993; Lee *et al.* 2005). To get a more detailed picture why 7,8-dihydroxyflavone is an efficient NQO1 inhibitor, docking and molecular dynamics studies were performed. The docking studies of 7,8-dihydroxyflavone in the catalytic site of human NQO1 showed a binding of this flavone stacked on the FAD isoalloxazine ring. The distance between the plane of the C ring of 7,8-dihydroxyflavone and the plane of FAD was in average 3.6 Å. This distance is comparable to the average distance found in the crystal structure between the planes of the FAD ring and the dicoumarol ring of 4 Å (Asher *et al.* 2006), which is a competitive inhibitor of NQO1 (Ernster *et al.* 1960). The competitive mode of NQO1 inhibition by 7,8-dihydroxyflavone was corroborated by

our experimental data (Fig. 2.3). Furthermore, 5 binding positions were defined after docking studies to the catalytic sites of the NQO1 dimer. The docking results show differences in the two catalytic sites of the NQO1 dimer. The X-ray 3D structure of the NQO1 dimer (1DXO) used for our docking and MD studies show small but clear differences in the binding site of the A and C chain. Based on these observations and on our docking and MD studies, the NQO1 protein probably has multiple 3D conformations. These different 3D conformations could possibly be regulated by physiological conditions (protein-protein interaction for instance). Docking in chain C led to energetically more favorable binding conformations, indicating that the orientation of the side chain of Tyr 128' is important for the interaction with 7,8-dihydroxyflavone. The importance of Tyr 128 has also been ascribed earlier by comparison of different crystal structures (Aarts *et al.* 1995; Faig *et al.* 2000) and site directed mutagenesis studies (Ma *et al.* 1992).

The most probable binding orientation of 7,8-dihydroxyflavone to the active site of NQO1 is mode 4, reported in this study (Fig. 5.4). The keto group of 7,8-dihydroxyflavone interacts with His 161 and Tyr 128'. The importance of these 2 residues for the interaction between NQO1 and 7,8-dihydroxyflavone in the catalytical site of NQO1 was shown with site directed mutagenesis studies (Aarts *et al.* 1995; Chen *et al.* 1999; Faig *et al.* 2000; Asher *et al.* 2006). Furthermore, binding mode 4 also explains why 7,8-dihydroxyflavone is the best NQO1 inhibitor among the flavonoids tested. The hydroxyl groups of this flavone at position 7 and 8 (Fig. 2.1) can form hydrogen bonds with Tyr 126', explaining the higher affinity to NQO1 than that of flavonoids without a 7,8-dihydroxy moiety.

Earlier, chrysin was reported to bind to a different site than dicoumarol, based on NQO1 enzyme proteolysis experiments and kinetic studies upon concomitant addition of dicoumarol and chrysin (Chen *et al.* 1993). Later, it was also reported based on docking studies of chrysin to NQO1 that the site of dicoumarol and chrysin binding were different (Chen *et al.* 1999). However, our study indicates that 7,8-dihydroxyflavone binds in a similar geometry as dicoumarol, as shown in Fig. 2.5. Superposition of the NQO1 in complex with dicoumarol (PDB accession code 2F1O) with the binding mode 4, shows very similar binding positions of these two competitive inhibitors at the active side of NQO1 (Fig. 2.6).



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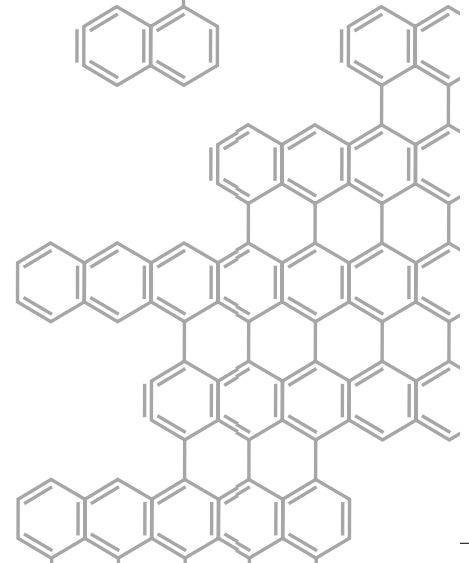
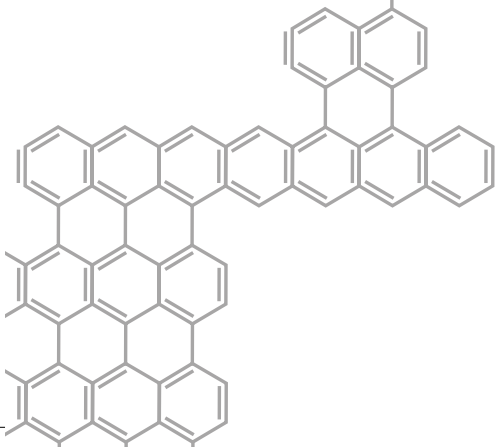
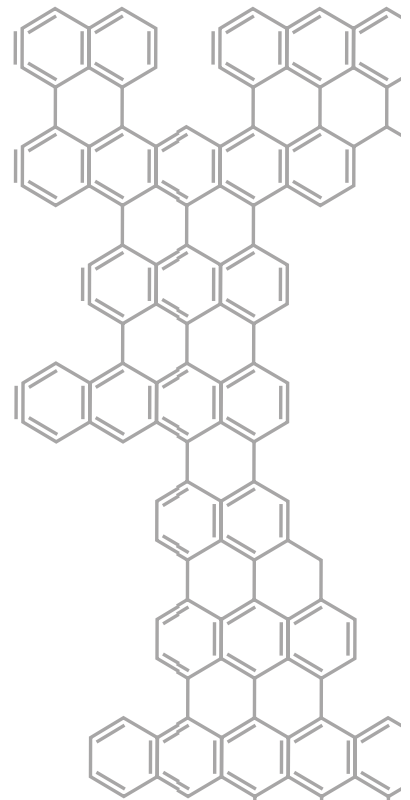
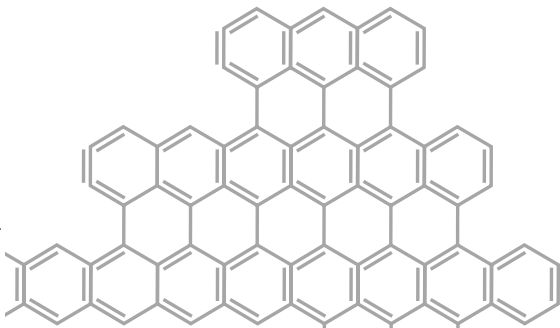
Figure 2.6: Comparison of the binding of 7,8-dihydroxy flavone and dicoumarol to the active site of NQO1. FAD, dicoumarol, 7,8-dihydroxyflavone and Tyr 128 are labeled for the crystal structure with PdB accession code 2F1O and for the binding mode 4 with bm4. Pictures were generated with VMD v1.8 (Humphrey *et al.* 1996).

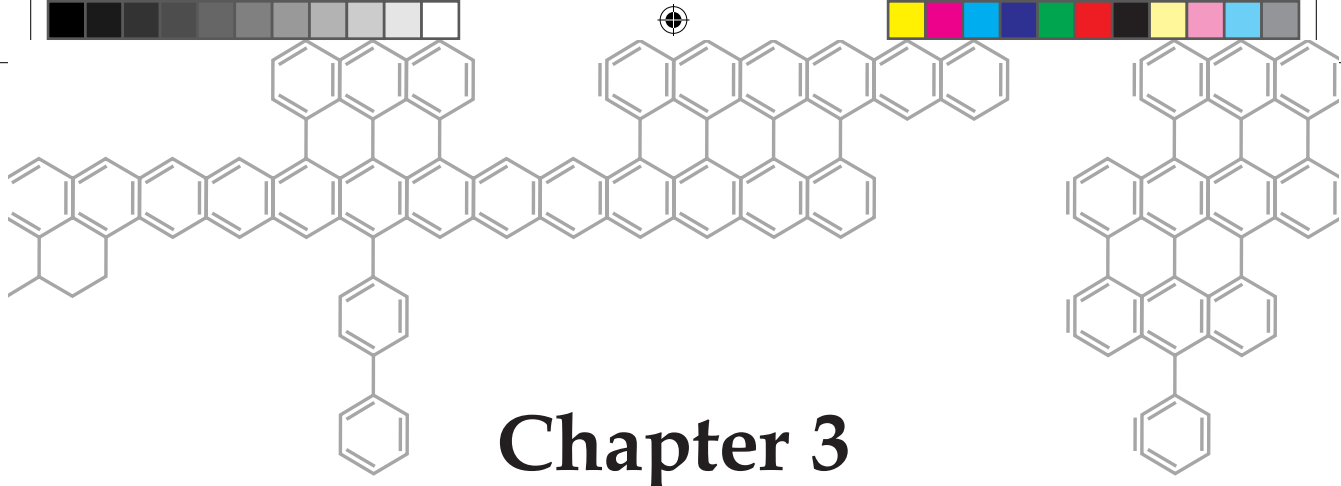
The orientation of Tyr 128' is different in the alignment, revealing the flexibility of this residue. Tyr 128 can adopt many different conformations, resulting in opening and closing of the binding site, as well as different interactions with substrates (Faig *et al.* 2000).

Altogether, the results of this study show a possible binding mode of 7,8-dihydroxyflavone to the active side of NQO1, explaining why this specific flavone is a good NQO1 inhibitor compared to other flavonoids. Furthermore, the results of this study show that docking alone is insufficient to describe binding-interactions in detail. More refined molecular dynamics studies are necessary to obtain a more detailed and reliable picture of the binding interactions (Alonso *et al.* 2006).

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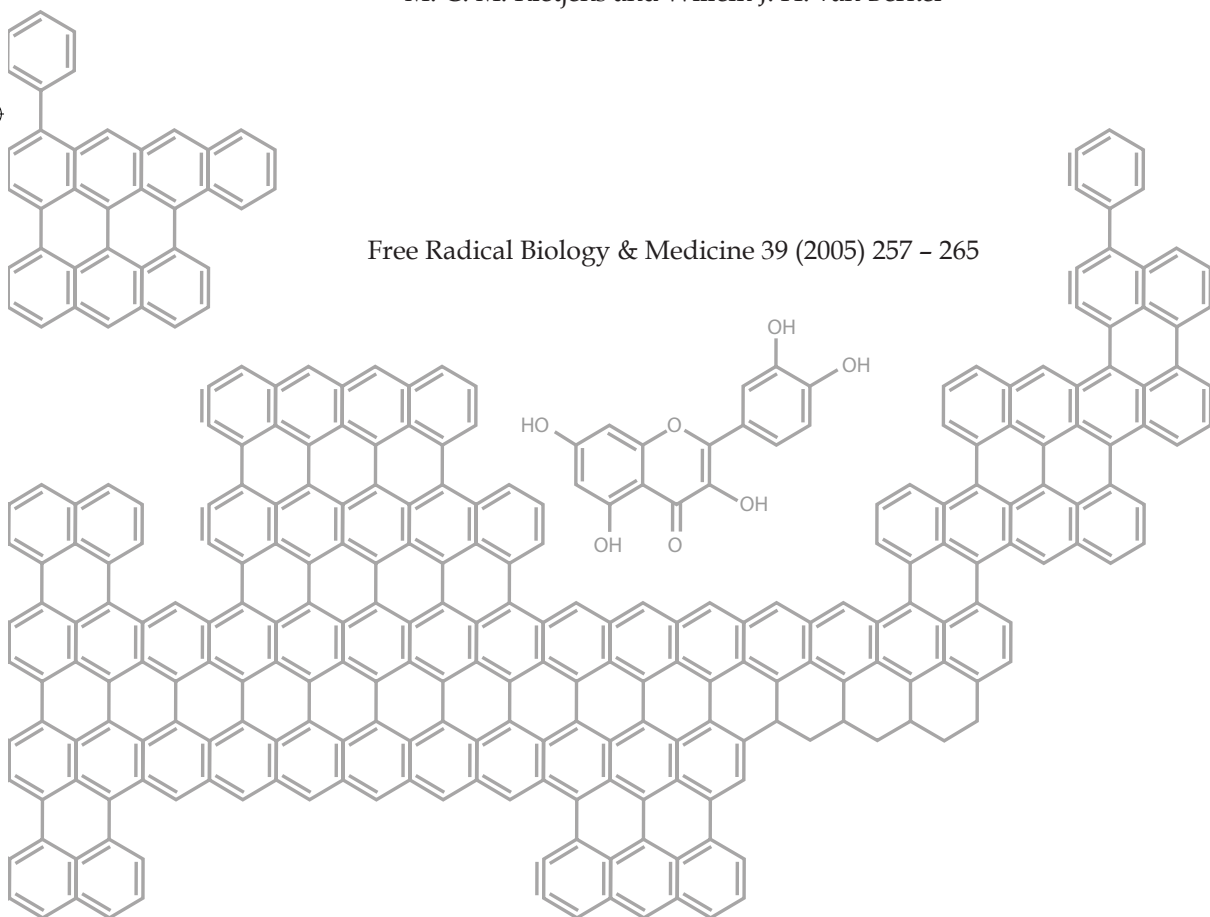




Human NAD(P)H: quinone oxidoreductase inhibition by flavonoids in living cells

Yee Y. Lee, Adrie H. Westphal, Laura H. J. de Haan, Jac M. M. J. G. Aarts, Ivonne M. C. M. Rietjens and Willem J. H. van Berkel

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Abstract

Procedures for assessing enzyme inhibition in living cells are an important tool to study the relevance of enzyme catalysed reactions and interactions in the human body. This paper presents the effects of flavonoids on NAD(P)H: quinone oxidoreductase 1 (NQO1) activity, by a newly developed method to measure NQO1 inhibition in intact cells. The principle of this method is based on the resorufin reductase activity of NQO1. The change in fluorescence in time was used to determine NQO1 activity in intact Chinese hamster ovary (CHO) cells genetically engineered to overexpress human NQO1. Applying this method to determine the inhibitory effects of reported *in vitro* NQO1 inhibitors (dicoumarol, 7,8 dihydroxyflavone, chrysin) showed that for all inhibitors tested, the IC_{50} in intact cells was at least 3 orders of magnitude higher than the IC_{50} in cell lysates. This result demonstrates that *in vitro* studies with purified NQO1 or with extracts from disrupted tissues are of limited value to obtain insight in the situation in living cells. Possible factors underlying this discrepancy are being discussed. For the first time, we determined NQO1 inhibition by flavonoids in cells without disruption of the cells or addition of cofactors, enabling the assessment of enzymatic activity and the interaction of modulators of enzymatic activity in an intracellular situation.

Introduction

Flavonoids are polyphenols naturally occurring in fruits, vegetables, grains, tea and wine. Epidemiological studies have indicated that flavonoids are preventive in coronary heart disease, stroke and certain cancer through their antioxidant, anti-inflammatory, anti-allergic and antiviral activities (Dajas *et al.* 2003; Peterson *et al.* 2003; Hannum 2004; Mennen *et al.* 2004). As such, flavonoids are generally considered to be beneficial to consumers' health and present one of the most important bioactive food components. On the other hand, there is considerable evidence that flavonoids may have pro-oxidant and DNA-damaging activity (Macgregor 1984; Awad *et al.* 2001; Gliszczynska-Swiglo *et al.* 2003; Walle *et al.* 2003). Many biologically active flavonoids appear to have effects on various proteins, including enzymes. *In vitro* inhibition studies have shown that flavonoids are able to inhibit the detoxification enzyme NAD(P)H:quinone oxidoreductase 1 (NQO1; EC 1.6.99.2) (Liu *et al.* 1990; Chen *et al.* 1993; Chen *et al.* 1999), pointing at a mechanism contradicting the proven beneficial properties of these phytochemicals. NQO1, also referred to as DT-diaphorase (Ernster and Navazio 1958), is a homodimeric, ubiquitous, cytosolic flavoprotein (Ross and Siegel 2004). It is able to perform two-electron reduction of quinones and nitro aromatics (Lind *et al.* 1982), thereby preventing the one electron reduction of quinones by cytochromes P450 and their redox cycling with molecular oxygen generating superoxide radicals, as well as their electrophilic activity resulting in covalent interaction with cellular macromolecules (Dinkova-Kostova and Talalay 2000). Thus, the obligatory two-electron reduction by NQO1 (Tedeschi *et al.* 1995; Faig *et al.* 2000) is responsible for the detoxifying properties of this enzyme. One of the main characteristics of NQO1 is its specific inhibition by dicoumarol. *In vitro* studies have shown that dicoumarol competes with the binding of NAD(P)H (Ernster *et al.* 1960). Dicoumarol inhibition is considered as an indicator for NQO1-mediated metabolism in biological systems (SantaCruz *et al.* 2004), for example in bioreductive activation or deactivation of antitumor quinones (Cullen *et al.* 2003; Dehn *et al.* 2004), in processes such as detoxification of quinones (Moran *et al.* 1999; Joseph *et al.* 2000), and in chemical induced mutagenicity studies (Joseph and Jaiswal 1998).

For the determination of NQO1 activity and the level of inhibition by different compounds, substrates like 2,6-dichlorophenolindophenol or menadione were used (Benson *et al.* 1980; Prochaska and Santamaria 1988). Furthermore, immunohistochemistry assays have been performed to localize the enzyme in different tissues (Siegel *et al.* 1998). However, so far all reported methods for measuring NQO1 activity rely on disruptive procedures for preparing subcellular fractions and thus do not necessarily represent the conditions of metabolism in the intact cell. Methods to assess the activity of NQO1 *in vivo* are needed to understand the role that flavonoids play in interaction with NQO1, taking availability of inhibitors and naturally present cofactors into account. Yet, there is no method reported to detect NQO1 activity and its inhibition inside intact cells.

To demonstrate the effects of flavonoids on NQO1 in living cells, we have developed a method to determine intracellular NQO1 activity. The principle of this method is based on the resorufin reductase activity of NQO1. Resorufin is a good substrate of NQO1 (Nims *et al.* 1984) and Sidhu and coworkers reported the use of a non-invasive scanning laser cytometry to determine cytochrome P450 activity on the basis of intracellular resorufin production in single cells where NQO1 was reported to reduce the resorufin product formed (Sidhu *et al.* 1993). Initial experiments showed that resorufin does not enter the cell and thus cannot be used as such to study intracellular NQO1 activity. Therefore we used a resorufin analogue, reported to be suitable for monitoring cell viability (Ishiyama *et al.* 1999). This compound *O*⁷-(isobutyloxycarbonyl)resorufin, is able to enter the cell and is subsequently hydrolysed by intracellular esterases to resorufin (Fig. 3.1).

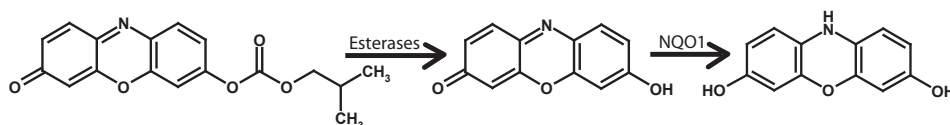


Figure 3.1 Intracellular two-step conversion of *O*⁷-(isobutyloxycarbonyl)resorufin. Resorufin is produced by hydrolysis of *O*⁷-(isobutyloxycarbonyl)resorufin by intracellular esterases and is further converted by cytosolic NQO1 to a non-fluorescent compound.

NQO1 activity can then be followed by the decrease of resorufin fluorescence. As a cellular model system we chose Chinese hamster ovary (CHO) cells, because i) it is a cell line expressing very low levels of NQO1 (Gustafson *et al.* 1996) and ii) we recently constructed a cell line (CHO-hNQO1) stably transfected with a human NQO1 expression vector (De Haan *et al.* 2002), conferring constitutive high level expression of the human NQO1 enzyme. The wild-type CHO cell line and the transfected CHO hNQO1 cell line have similar genetic and physiological background and only differ specifically in their level of NQO1 expression. The transfected cell line has the additional advantage of stable NQO1 expression, because the NQO1 expression is under control of a viral promoter instead of the natural NQO1 gene promoter.

The objective of this study was to determine the interaction between flavonoids and the important cellular detoxification enzyme NQO1 in living cells, supporting or contradicting to the supposing beneficial effects of flavonoids. In this work we demonstrate that reported potent inhibitors of NQO1 activity *in vitro* show a much lower level or even no inhibition in intact cells.

Materials & Methods

Chemicals

DMEM/F12 medium, Hanks' balanced salt solution (HBSS), trypsin, foetal calf serum (FCS), phosphate buffered saline (PBS), gentamicin and zeocin were purchased from Gibco Invitrogen Corporation (Paisley, Scotland). Dimethyl sulfoxide (DMSO) and quercetin were obtained from Acros Organics (New Jersey, USA). Isorhamnetin, chrysin, 7-hydroxyflavone and 7,8-dihydroxyflavone were purchased from Extrasynthese (Genay Cedex, France). Dicoumarol, resorufin, O⁷-(isobutyloxycarbonyl)resorufin and uridine 5'-diphosphoglucuronic acid (UDPGA) were purchased from Sigma (St. Louis, MO) and NADPH was obtained from Roche (Mannheim, Germany). For all standards and substrates a fresh stock solution in DMSO was prepared for each experiment.

Cell lines

Chinese hamster ovary (CHO) cells were purchased from the American Type Culture Collection (Manassas, VA). CHO cells were cultured in DMEM/F12 medium supplemented with 10% FCS and 50 µg/mL gentamicin. The cells were maintained in a humidified atmosphere with 5% CO₂ at 37°C. The CHO-hNQO1 cells were stably transfected with the expression vector pcDNA3.1/Zeo(+) carrying the human NQO1 cDNA sequence strain CHO-hNQO1-5 as described previously (De Haan *et al.* 2002). The culture medium of the CHO-hNQO1 cells was the same as for the wild-type CHO cells, containing in addition 200 µg/mL zeocin.

NQO1 activity in disrupted cells

NQO1 activity in disrupted cells was assayed fluorometrically as the dicoumarol inhibitable fraction of resorufin reduction in the cell cytosol (Sidhu *et al.* 1993). Cells were trypsinised and resuspended in buffer, containing 20 mM Tris-HCl and 2 mM EDTA pH 7.4. Cells were disrupted in three cycles of freezing and thawing using liquid nitrogen and a 37°C waterbath. After centrifugation for 5 min at 9,000 g an appropriate amount of cell lysate was added to the reaction mixture, containing 100 µM NADPH in a final volume of 200 µL PBS (pH 7.4). The reaction was initiated by addition of 10 µL of a 10 µM resorufin stock solution in PBS and 10% DMSO, resulting in a final concentration of 500 nM resorufin and 0.5% DMSO in the reaction mixture. The reduction of resorufin was monitored at $\lambda_{em}/\lambda_{ex}$ 590 nm/522 nm in a microplate reader at room temperature for 2 min in the absence or presence of 10 µM dicoumarol. The rate of resorufin reduction was calculated by comparing the change of fluorescence as a function of time, relative to the fluorescence of a known amount of resorufin. The results are expressed in nmol resorufin reduced per minute per mg cytosolic protein. The inhibition constants for flavonoids and dicoumarol were derived from Dixon plots. Protein content was measured using the BCA protein reagent kit from Pierce (Rockford, USA) as described elsewhere (Shihabi and Dyer 1988).

Glucuronidation

Conjugation reactions of resorufin or O^7 -(isobutyloxycarbonyl)resorufin with UDP glucuronosyltransferase (UGT) were tested with disrupted wild-type CHO cells. Cells were trypsinised and disrupted by freezing and thawing and an appropriate amount of this cell lysate was added to the reaction mixture, containing 1 μ M resorufin or O^7 -(isobutyloxycarbonyl)resorufin. The fluorescence of the reaction mixture was monitored at $\lambda_{em}/\lambda_{ex}$ 590 nm/522 nm in a microplate reader at room temperature for 2 min in the absence or presence of 300 μ M of the donor substrate UDPGA.

NQO1 activity in intact cells

For determination of NQO1 activity in intact cells, cell suspensions (2×10^5 cells/mL) were plated in culture medium in 96-wells-plates (100 μ L/well) and incubated for 24 hours to allow attachment of the cells on to the bottom of the wells and the formation of a confluent monolayer. Next, the culture medium was removed and cells were rinsed twice with PBS. The reaction was initiated by addition of O^7 -(isobutyloxycarbonyl)resorufin (5 μ M final concentration in PBS) in the absence or presence of inhibitors. The increase of fluorescence intensity through hydrolysis of O^7 -(isobutyloxycarbonyl)resorufin by cellular esterases to resorufin (Ishiyama *et al.* 1999), was monitored with a microplate reader at room temperature for 5 min at $\lambda_{em}/\lambda_{ex}$ 590 nm/522 nm. The increase in resorufin fluorescence in CHO-hNQO1 cells was compared to the fluorescence increase in dicoumarol inhibited CHO-hNQO1 cells and wild-type CHO cells to provide an independent proof of the NQO1-mediated nature of the observed effect. The rate of resorufin production was quantified by comparison of the time dependent increase in fluorescence intensity to the fluorescence of a known amount of resorufin. The NQO1 activity was calculated as the difference in time-dependent fluorescence increase of dicoumarol-inhibited CHO-hNQO1 cells and non inhibited CHO-hNQO1 cells. Specific enzyme activities are expressed in pmol resorufin produced per min per monolayer or in nmol resorufin produced per minute per mg protein. Protein contents of monolayers of CHO cells were determined using the BCA protein reagent kit from Pierce (Rockford, USA) (Shihabi and Dyer 1988). IC_{50} data were measured using 5 μ M of O^7 -(isobutyloxycarbonyl)resorufin and obtained by variation of inhibitor concentration in the incubation.

Cytotoxicity

Cytotoxicity of the substrates was tested, by using the lactate dehydrogenase (LDH) assay with minor adaptations for 96-well-plates (Mitchell and Acosta 1980). Briefly, cells were plated at a density of 10^4 cells per well in a 96 well-plate for 24 hours before exposure. Subsequently, for testing the cytotoxicity of resorufin, 200 μ L of culture medium containing different concentrations of resorufin were added. The DMSO concentration in the culture medium was kept constant at 0.5%. After 24 hours of exposure, culture medium was collected, cells were lysed and LDH activity was

measured in the culture medium fraction and in the cell lysate. For testing the cytotoxicity of *O*⁷-(isobutyloxycarbonyl)resorufin the LDH assay was modified, because initial experiments showed that *O*⁷-(isobutyloxycarbonyl)resorufin is hydrolysed to resorufin, when added to the culture medium. Therefore the LDH viability assay for *O*⁷-(isobutyloxycarbonyl)resorufin was performed in PBS, in which no hydrolysis of *O*⁷-(isobutyloxycarbonyl)resorufin occurs. Cells in 96-well-plates were exposed to 200 μ L of PBS containing different concentrations of *O*⁷-(isobutyloxycarbonyl)resorufin and incubated for 24 hours. After incubation PBS was collected, cells were lysed and LDH activity was measured in the collected PBS and in the cell lysate.

Results

Effect of inhibitors on NQO1 activity in disrupted cells

Inhibition studies of NQO1 activity in disrupted cells were performed for comparison with inhibition studies in intact cells. In the presence of NADPH, resorufin is reduced by NQO1 in the cytosolic fraction of CHO-hNQO1 cells. This reaction can be followed by means of the linear time-dependent loss of resorufin fluorescence. When either NADPH or cell lysate was omitted, no decrease in fluorescence was observed. The addition of 10 μ M dicoumarol completely prevented the loss of resorufin fluorescence. A linear response between resorufin concentration and resorufin fluorescence was observed at concentrations between 0–2 μ M. Resorufin concentrations higher than 2 μ M did not give a linear fluorescence response with increasing concentration. Fig. 3.2 shows the dependency of the NQO1 activity on NADPH.

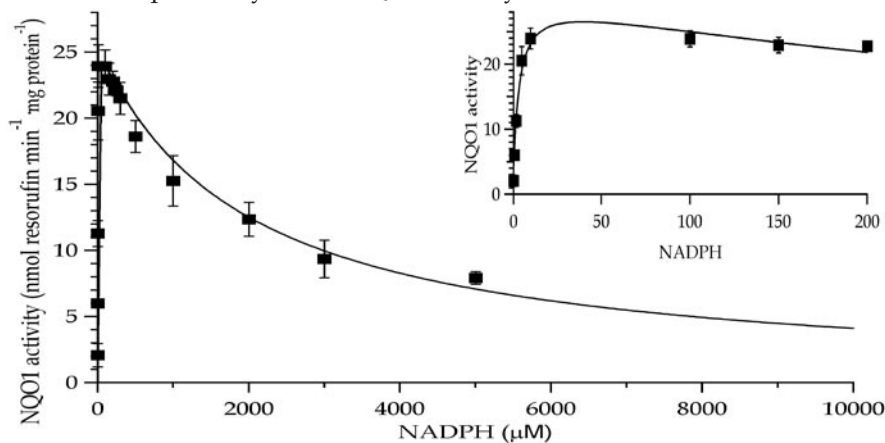


Figure 3.2: NADPH dependence of resorufin reduction by lysed CHO-hNQO1 cells. The reaction was performed with lysed CHO-hNQO1 cells and 500 nM resorufin. NQO1 activity is expressed in nmol resorufin reduced per minute per mg protein and decreases above 100 μ M NADPH with increasing NADPH concentration. Data are fitted to the equation for substrate inhibition $v = \frac{V_{\max} \cdot [NADPH]}{K_M + [NADPH] + \frac{[NADPH]^2}{K_I}}$ and presented as mean \pm standard deviation (n=4).

At NADPH concentrations higher than 100 μM a decrease in NQO1 activity was observed. Inhibition up to 70% of NQO1 activity was found at a NADPH concentration of 5 mM. The resorufin reduction can be described with the equation for substrate inhibition (Fig. 3.2 legend). The estimated apparent K_M value (K'_M) for NADPH was $2.2 \pm 0.4 \mu\text{M}$. Subsequently, by varying the concentration of resorufin at a constant level of the second substrate NADPH (100 μM) the estimated K'_M value for cytosolic NQO1 activity was $2.5 \pm 0.3 \mu\text{M}$ for resorufin, with an apparent V_{max} (V'_{max}) at 100 μM NADPH of $77 \pm 2 \text{ nmol resorufin per min per mg protein}$.

The inhibitory effect of dicoumarol and several flavonoids (Fig. 3.3) on the activity of cytosolic CHO hNQO1 is summarized in Table 3.1.

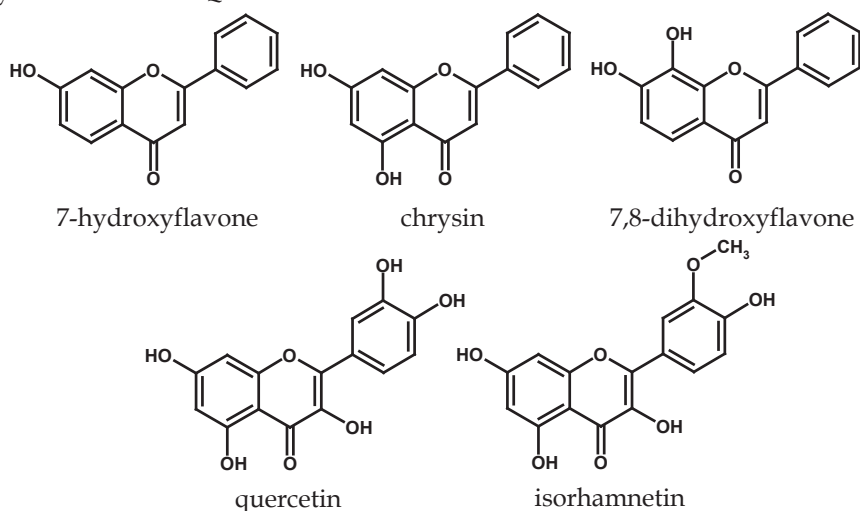


Figure 3.3: Chemical structures of flavonoids tested in this study.

Table 3.1: Competitive inhibition constants (K_i) of human NQO1 activity in lysed CHO-hNQO1 cells by different NQO1 inhibitors

| Inhibitor | $K_i(\text{nM})$ |
|----------------------|------------------|
| dicoumarol | 0.5 ± 0.1 |
| 7,8-dihydroxyflavone | 16 ± 2 |
| chrysin | 17 ± 3 |
| 7-hydroxyflavone | 309 ± 22 |
| isorhamnetin | 1030 ± 231 |
| quercetin | 6190 ± 840 |

As expected, dicoumarol showed to be a very strong inhibitor of NQO1 activity. The inhibition constant (K_i) for competitive inhibition towards NADPH was found to be 0.5 nM with lysed CHO-hNQO1 cells. The most potent flavonoid inhibitors of NQO1 activity showed to be 7,8-dihydroxyflavone and chrysin. With resorufin as a substrate a K_i of 16 ± 2 nM for 7,8-dihydroxyflavone and a K_i of 17 ± 3 nM for chrysin was observed. The inhibition constants for 7-hydroxyflavone and isorhamnetin were higher being 309 ± 22 nM for 7-hydroxyflavone and $1.0 \pm 0.2 \mu\text{M}$ for isorhamnetin. Quercetin showed the weakest inhibition with a K_i of $6.2 \pm 0.8 \mu\text{M}$. Fig. 3.4 shows that 7,8-dihydroxyflavone inhibits NQO1 activity in CHO-hNQO1 cytosol competitive with NADPH.

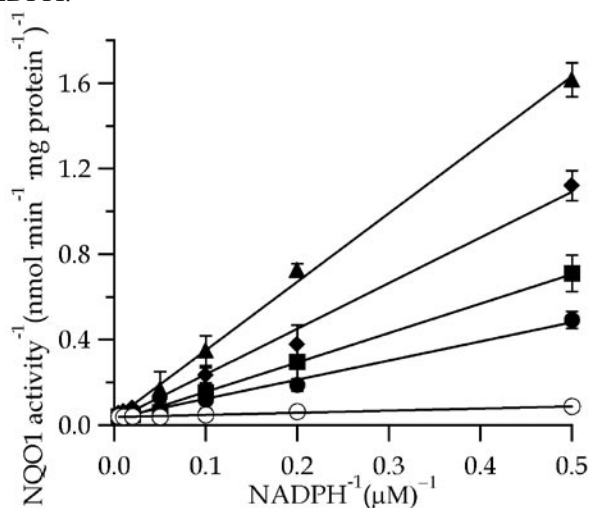


Figure 3.4: Inhibition of hNQO1 activity in CHO-hNQO1 cell lysates by 7,8-dihydroxyflavone. Conditions: PBS, 500 nM resorufin, an amount of CHO-hNQO1 cell lysates containing 2 μg of protein and varying concentrations of 7,8-dihydroxyflavone (-○-0 μM, -●-0.2 μM, -■-0.3 μM, -◆-0.4 μM, -▲-0.5 μM) and NADPH. Data are presented as mean ± standard deviation (n=4).

All flavonoids tested showed this competitive mode of inhibition.

NQO1 activity in intact cells

To detect NQO1 activity in intact cells we used *O*⁷-(isobutyloxycarbonyl)resorufin which, in contrast to resorufin, was found to be readily taken up by the cells. Inside the cells, this compound is hydrolysed by esterases into resorufin, the actual substrate of NQO1. Therefore, we first investigated whether these two compounds are non-toxic to the cells by means of the LDH leakage assay, which clearly showed that the viability of the cells is 100% up to a concentration of at least 20 μM resorufin and *O*⁷-(isobutyloxycarbonyl)resorufin (data not shown).

To optimise the substrate availability for the intracellular esterases different concentrations of O^7 -(isobutyloxy carbonyl)resorufin were tested and the resulting fluorescence intensity of resorufin was determined. A concentration of $5 \mu\text{M}$ O^7 -(isobutyloxy carbonyl)resorufin resulted in a resorufin production of 6.0 ± 0.2 pmol resorufin per min per monolayer wild-type CHO cells. Higher concentrations of O^7 -(isobutyloxy carbonyl)resorufin did not show higher rates of resorufin production. Therefore, we concluded that $5 \mu\text{M}$ O^7 -(isobutyloxy carbonyl)resorufin was sufficient for the esterases in CHO cells to reach their maximum rate of 6 ± 0.2 pmol resorufin produced per min per monolayer. Furthermore, the production rate of resorufin in intact cells and disrupted cells appeared to be equal (data not shown). Possible conjugation reactions of O^7 -(isobutyloxy carbonyl)resorufin or resorufin by UDP glucuronosyltransferases (UGT) inside cells, leading to less resorufin fluorescence produced in time, were tested by adding the glucuronosyl group donation substrate UDPGA. No change in resorufin fluorescence was monitored in lysed wild-type CHO cells with and without addition of the donor substrate UDPGA. No glucuronidation of O^7 -(isobutyloxy carbonyl)resorufin or resorufin was observed (data not shown).

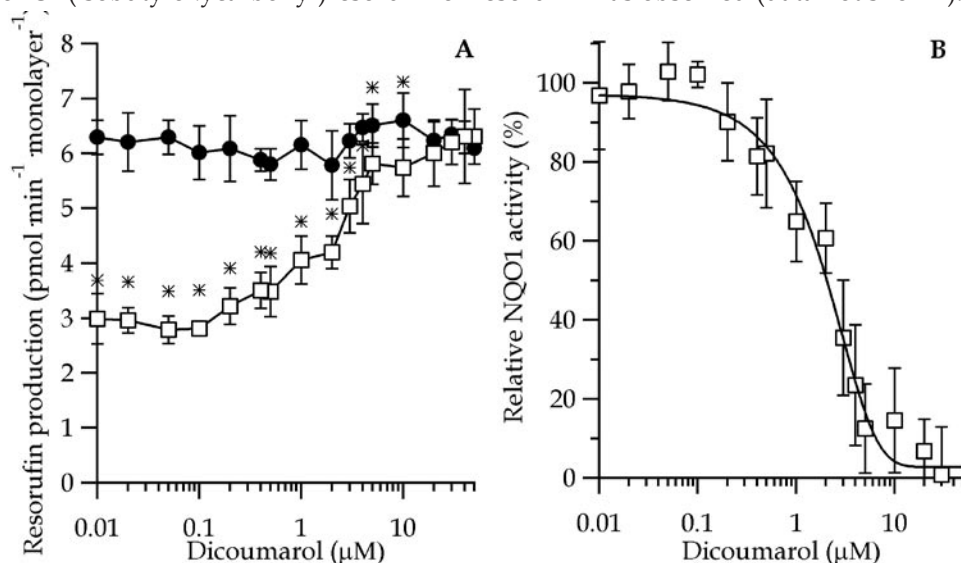


Figure 3.5: Effects of dicoumarol on resorufin production in living CHO cells. **A**, Resorufin production in dependence of dicoumarol concentrations in wild-type (-□-) and CHO-hNQO1 (-●-) cells. Activities of CHO-hNQO1 cells are significantly different (*) from wild-type CHO cells when $P < 0.05$, according to the Student's *t*-test. **B**, Dose response curve of dicoumarol in intact CHO-hNQO1 cells.

The relative NQO1 activity is the percentage of NQO1 activity at different dicoumarol concentrations compared to the NQO1 activity in CHO-hNQO1 cells unexposed to dicoumarol. Data are presented as mean \pm standard deviation ($n=4$).

Fig. 3.5A shows the resorufin production in wild-type CHO cells compared to the resorufin production in CHO-hNQO1 cells at different concentrations of dicoumarol. From these data, the NQO1 activity in CHO-hNQO1 cells can be calculated by subtracting the value of resorufin production in wild-type CHO cells by the value of resorufin production in hNQO1-CHO cells, when no dicoumarol was added. The intracellular NQO1 activity in CHO-hNQO1 cells was determined to be 3.1 ± 0.1 pmol resorufin reduced per min per monolayer or 0.08 nmol resorufin reduced per min per mg protein. Furthermore, Fig. 3.5A also presents the effect of dicoumarol in the wild-type CHO and CHO-hNQO1 cells. With increasing dicoumarol concentration a concentration dependent increase in the resorufin production in CHO-hNQO1 cells but no change in resorufin production in wild-type CHO cells was observed. Increasing resorufin production in CHO-hNQO1 cells started at a concentration of dicoumarol of around 0.1 μ M. The resorufin production increased up to 10 μ M dicoumarol and at around 30 μ M dicoumarol, the production rate of resorufin in CHO-hNQO1 cells reached the level of the wild-type CHO cells. Dicoumarol, a specific inhibitor of NQO1, had no effect on resorufin production in the wild-type CHO cells, but increased the resorufin production in CHO-hNQO1 cells, which presents additional evidence that inhibition of NQO1 in CHO-hNQO1 cells is causing the increased resorufin production. Under the conditions applied, a concentration of 30 μ M dicoumarol is needed to obtain complete NQO1 inhibition in intact CHO-hNQO1 cells. Fig. 3.5B shows these results as a dose response curve of dicoumarol-mediated NQO1 inhibition. In CHO-hNQO1 cells the IC_{50} value derived from this plot was 1.6 μ M.

3

Flavonoid inhibition of NQO1 in intact cells

To examine intracellular inhibition of NQO1 activity in CHO-hNQO1 cells, several flavonoids able to inhibit NQO1 in cell lysates (Table 3.1) were tested for their inhibitory capacity, using the newly developed assay in living cells.

It was found that chrysin, 7-hydroxyflavone and quercetin, with concentrations up to 100 μ M and isorhamnetin with concentration up to 10 μ M, respectively, were unable to inhibit intracellular NQO1 activity in intact CHO-hNQO1 cells. Applying higher concentrations of these compounds was not possible due to their limited solubility. Only 7,8-dihydroxyflavone showed an inhibitory effect on NQO1 activity in intact cells (Fig. 3.6).

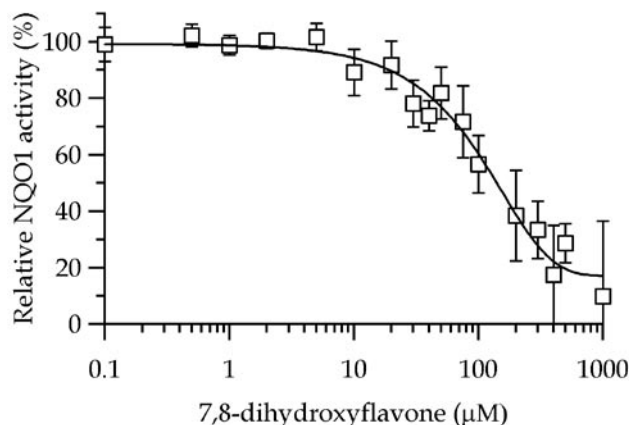


Figure 3.6: Dose-response curve of 7,8-dihydroxyflavone mediated inhibition of NQO1 activity in intact CHO-hNQO1 cells. The relative NQO1 activity is expressed as the percentage of NQO1 activity at different 7,8-dihydroxyflavone concentrations compared to the NQO1 activity in CHO-hNQO1 cells unexposed to 7,8-dihydroxyflavone. Data are presented as mean \pm standard deviation ($n=4$).

The inhibition of 7,8-dihydroxyflavone was relatively weak. The IC_{50} value derived from the dose response curve was 133 μ M. Inhibition of resorufin production in wild-type CHO cells was not observed with all inhibitors tested.

Discussion

NQO1 is a major cellular detoxification enzyme. *In vitro* studies have shown that the activity of NQO1 is inhibited by flavonoids, which would be contradicting to the reported beneficial effects of these phytochemicals. Here we show for the first time that inside living cells, inhibition of NQO1 by flavonoids does not occur at concentrations, showing inhibition in *in vitro* studies.

To determine the interaction between NQO1 and flavonoids in living cells, we developed a method based on the dicoumarol-inhibitable resorufin reductase activity of NQO1. To inhibit the NQO1 activity in living CHO-hNQO1 cells, a high concentration of dicoumarol (1.6 μ M for 50% inhibition) was needed, whereas the inhibition constant of dicoumarol *in vitro* is 0.5 nM. The dicoumarol inhibition was quantified as the difference in resorufin production between inhibited and uninhibited CHO-hNQO1 cells. Dicoumarol did not influence resorufin production in wild-type CHO cells, thus verifying that indeed no enzymatic activities other than NQO1 are taking part in the reaction in the CHO-hNQO1 cells, which differ only from wild-type CHO cells in the constitutive expression of the human NQO1 enzyme as the only genetic difference. In line with the observed decreased dicoumarol inhibition in living cells, several flavonoids, known to inhibit NQO1 activity in *in vitro* systems, did not inhibit the enzyme activity in living CHO-hNQO1 cells, indicating that inhibition studies with

purified NQO1 or disrupted tissue do not necessarily reflect the situation in living cells. Among the flavonoids tested, 7,8-dihydroxyflavone was the only compound that inhibited NQO1 activity in living cells. Again, however, a high concentration (133 μM for 50% inhibition) was needed to inhibit NQO1 activity in intact cells, whereas the inhibition constant of 7,8-dihydroxyflavone *in vitro* is 16 nM. For chrysin, 7-hydroxyflavone, quercetin and isorhamnetin no inhibition was observed in living cells at concentrations up to the solubility limit of the compound.

We suggest that the ineffectiveness of *in vitro* NQO1 inhibitors in living cells is due to several cellular factors. One of them is the high NAD(P)H concentration inside cells. Dicoumarol and flavonoids compete with NAD(P)H for binding to NQO1 (Fig. 4), thereby inhibiting its activity (Ernster *et al.* 1960; Chen *et al.* 1993). Three dimensional structural data showed that the binding position of these inhibitors overlaps with the binding position occupied by the pyridine nucleotide (Li *et al.* 1995; Chen *et al.* 1999). This, and the fact that the enzyme acts according to a ping-pong mechanism (Bianchet *et al.* 1999), implies that binding of dicoumarol and flavonoids to the oxidized enzyme inhibits the electron transfer from NAD(P)H to FAD (Huang *et al.* 1987). Consequently, a high NAD(P)H concentration inside cells prevents the inhibition of NQO1 activity by dicoumarol and other competitive inhibitors. Nagele (Nagele 1995) reported a concentration of 0.7 mM NADPH in CHO cells. Assuming competitive inhibition and K_i values of 0.5 nM (dicoumarol) and 16 nM (7,8-dihydroxyflavone), respectively, the estimated theoretical IC_{50} value with 0.7 mM NADPH is 0.16 μM for dicoumarol inhibition and 5 μM for 7,8-dihydroxyflavone inhibition. These theoretical values are only 10-26 fold lower than the experimentally determined IC_{50} values, which are 1.6 μM for dicoumarol and 133 μM for 7,8-dihydroxyflavone, respectively. To verify this calculation we estimated the NADPH concentration, according to the method of Zhang *et al.* (Zhang *et al.* 2000), to be 0.9 mM NADPH inside CHO cells (data not shown). Our results together with literature data strongly suggest that indeed the NADPH concentration inside cells is the main factor contributing to the discrepancy between intracellular and *in vitro* inhibition studies of NQO1. Still, this estimation does not take the NADH concentration inside the cell into account. The amount of NADH inside cells can also modulate the intracellular NQO1 activity, as well as the interaction with inhibitors, since NQO1 is also able to use NADH as a reducing cofactor, with a K_M of 86 μM (Ma *et al.* 1992) and thus providing a possible explanation to the difference between the theoretical values and the experimentally determined IC_{50} values. The contribution of these naturally present cofactors inside cells to the insensitivity of NQO1 against inhibitors plays an important role but is neglected in *in vitro* studies.

Another factor, which possibly contributes to the prevention of NQO1 inhibition in living cells, is the binding of inhibitors to cellular compounds and/or their conversion to metabolites. Dicoumarol has been reported to be extensively protein bound (Hulse *et al.* 1981; Madari *et al.* 2003). Quercetin can be metabolised to isorhamnetin and conjugated with glucuronic acid and sulfate (Manach *et al.* 1998; Murota and

Terao 2003), whereas its oxidized form covalently binds to glutathione, DNA and protein (Boersma *et al.* 2002; Awad *et al.* 2003; Walle *et al.* 2003). These naturally occurring cellular processes would result in a decrease in the concentration of the inhibitors and in a lowering of NQO1 inhibition in intact cells as compared to *in vitro* inhibition studies.

Although cellular uptake of flavonoids is dependent on the flavonoid and the cell type (Spencer *et al.* 2004), chrysin was predicted to have favourable membrane transport properties (Walle *et al.* 1999). Accordingly, high membrane permeability of chrysin in the human colonic cell line Caco-2 as a model of the human intestine was found (van der Woude *et al.* 2004). Furthermore, quercetin uptake is shown in Caco 2-, HT29-, HepG2-, DHD/K12/TRb, H4IIEwt and IEC-6-cells (Murota and Terao 2003; van der Woude *et al.* 2004). Also rapid dicoumarol uptake has been reported earlier in isolated rat hepatocytes (Wosilait *et al.* 1981). These data suggest that the uptake of inhibitors is unlikely to contribute to the discrepancy between intracellular and *in vitro* inhibition.

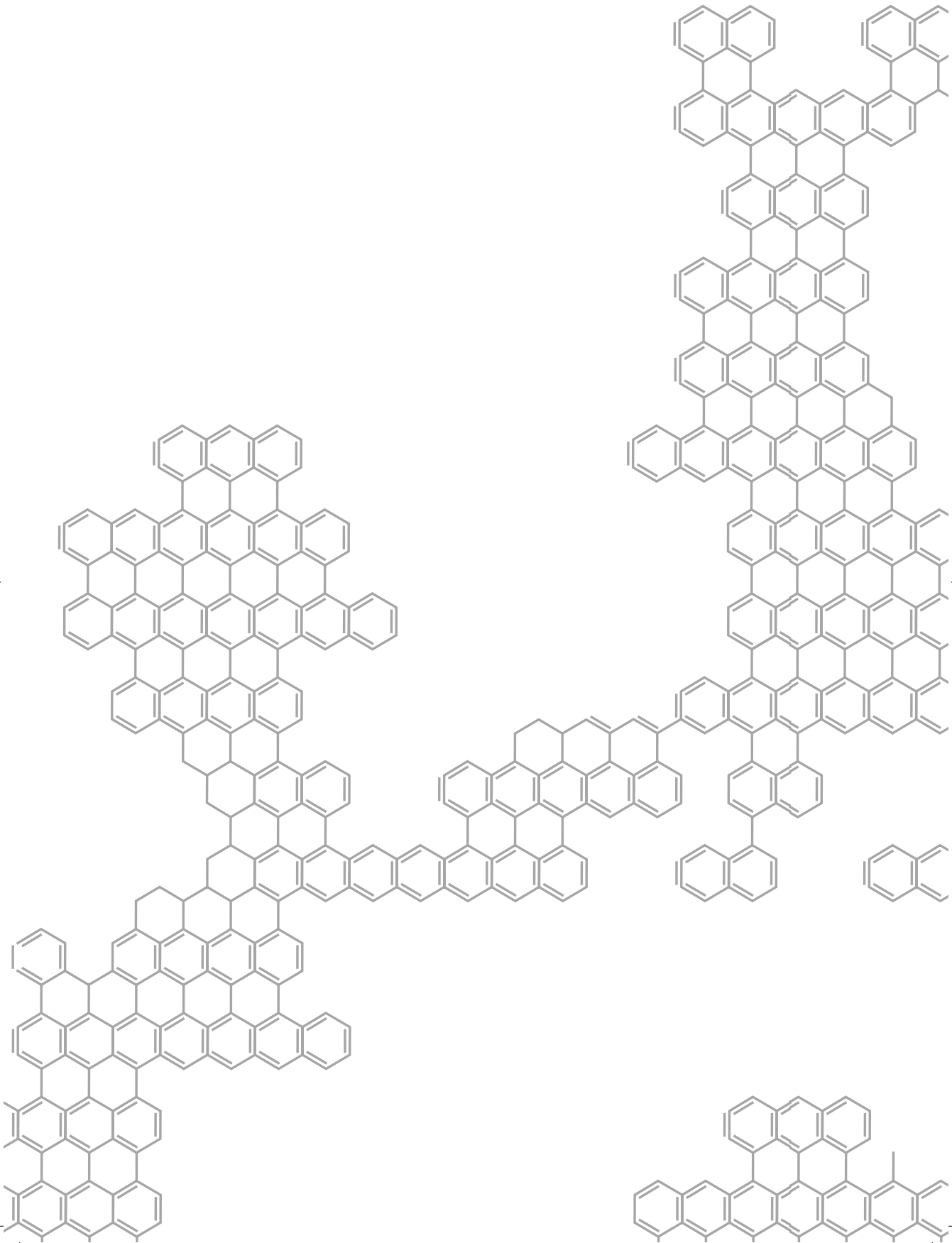
In spite of the fact that flavonoids are omnipresent in our diet, the availability of flavonoids at concentrations above 100 μM , possibly inhibiting NQO1 activity in human tissue, is questionable. Human pharmacokinetic studies reported maximal plasma concentrations of chrysin from 12 to 64 nM after a non toxic oral dose of chrysin (Walle *et al.* 2001). 7-Hydroxyflavone and 7,8-dihydroflavone are synthetic flavonoids, which are not present naturally in our diet. Reported concentrations of quercetin and isorhamnetin in human serum and plasma reach levels up to 5 μM after supplemented diet at non-toxic supplementation levels (Hollman *et al.* 1996; Olthof *et al.* 2000; Ishii *et al.* 2003). These observations together with our results strongly indicate that NQO1 inhibition by the tested flavonoids is improbable *in vivo*. Unclear and not to be ruled out is a possible local NQO1 inhibition due to accumulation of the flavonoid in the intestine after a high flavonoid supplementation.

The fate of enzyme inhibitors inside intact cells is complex and until now not fully understood. Future studies will provide more insight into this complex interplay. All mentioned factors of cellular processes and cellular defence mechanisms are likely to contribute to the discrepancy in inhibition observed in cell free extracts and in intact cells, pointing to the importance of inhibition studies in living cells reflecting the intracellular situation. More detailed investigations towards the actual fate of compounds inside cells should be done, to be able to predict their effects in living cells.

This study clearly indicates the relevance of our newly developed method to measure inhibition of NQO1 in living cells. This method is based on the resorufin reductase activity of NQO1, which appeared to be highly specific for NQO1 with a K_M value of 2.5 μM . Other enzymes can also react with resorufin but with much higher K_M values (NADPH-cytochrome P450 reductases: $K_M = 125 \mu\text{M}$ (Dutton *et al.* 1989; Balvers *et al.* 1992), phenol sulfotransferase: $K_M = 50 \mu\text{M}$ (Beckmann 1991)). Reactions of

resorufin with other enzymes do not interfere with the interaction between flavonoid and NQO1, because the NQO1 activity as defined in this study is the dicoumarol-inhibitable resorufin reducing activity, which is specific for NQO1. Nevertheless, our newly developed method is not yet suitable to be used as routine analysis for measuring intracellular NQO1 activity in different cell types. This is due to as yet unidentified variations of cellular context in different cell types that may lead to indefinable and not comparable reactions of resorufin with e.g. high levels of sulfotransferases or one electron reductases, autooxidation, or interaction of superoxide with reduced resorufin (Dutton *et al.* 1989). Because resorufin is not taken up by the cells, it has to be produced inside the cell via hydrolysis of O⁷-(isobutyloxycarbonyl)resorufin by intracellular esterases. This creates a system depending on initial reactions of esterases to enable measuring of NQO1 activity. The maximal rate of resorufin reduction by NQO1 in intact cells was lower, compared to the maximal rate for disrupted cells. A possible explanation for this observation is non-saturating substrate conditions in the assay due to limited intracellular esterase activity. Our results show that the maximal rate of intracellular resorufin production by esterase activity was reached. The uptake of O⁷-(isobutyloxycarbonyl)resorufin was not limiting the resorufin production inside cells. In addition, glucuronidation of resorufin or O⁷-(isobutyloxycarbonyl)resorufin, leading to reduced resorufin fluorescence produced in time could be excluded. Furthermore, Spencer and Rifkind (Spencer and Rifkind 1990) reported inhibition of resorufin reduction by NQO1 in pig and rat liver cytosol by NADPH at concentrations above 200 μM. This observation together with the estimated high NADPH concentration inside the cells indicate that the low level of NQO1 activity in intact CHO-hNQO1 cells as compared to the activity in cell free extracts of the same cells, is partially due to the high intracellular NADPH concentration leading to substrate inhibition. Taken together, insufficient intracellular esterase activity in combination with intracellular inhibition of NQO1 activity by NADPH are factors contributing to the lower NQO1 activity inside intact cells compared to *in vitro* results. However, a low NQO1 activity in living cells does not intervene with the goal of this study to measure intracellular inhibition of NQO1 activity with this method.

In conclusion, this study demonstrates that *in vitro* inhibition studies with purified NQO1 or disrupted tissues do not reflect the situation in living cells and that inhibition of NQO1 in the human body is not so easily achieved as assumed on the basis of *in vitro* studies.





Chapter 4

Shifted concentration dependency of
EpRE- and XRE-mediated gene expression
points at monofunctional EpRE-mediated
induction by flavonoids at physiologically
relevant concentrations



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Rietjens and Jac M. M. J. G. Aarts

Submitted to *Toxicology in Vitro*



Abstract

Flavonoids, important bioactive compounds, omnipresent in the human diet, are reported to be bifunctional inducers. These phytochemicals are able to induce XRE- and EpRE-mediated gene expression, resulting in the induction of phase 1 and phase 2 biotransformation enzymes. To test whether flavonoid-induced EpRE-mediated gene expression could be the result of upstream XRE-mediated gene expression, several flavonoids were tested for their ability to induce XRE- and EpRE-mediated gene expression using two stably transfected reporter gene cell lines constructed in the same mouse Hepa-1c1c7 hepatoma background. Although classified as bifunctional inducers, all flavonoids were found to induce EpRE- and XRE-mediated gene expression in a different concentration range, which presents an issue not considered by the current definition of a bifunctional inducer. At physiological relevant concentrations, the induction of gene expression via the EpRE transcriptional enhancer element is dominant, leading in particular to elevated levels of detoxifying phase 2 enzymes. Furthermore, these results strongly suggest that EpRE-mediated gene expression induced by flavonoids is not a downstream reaction of XRE-mediated gene expression.

Introduction

Flavonoids present important bioactive food components in the human diet and have been suggested to exert various beneficial effects in a multitude of diseases, including cancer, cardiovascular disease, neurodegenerative disorders, diabetes, and osteoporosis (Scalbert *et al.* 2005). These natural food components can exert protective effects by selectively inhibiting or increasing the expression level or the biological activity of key proteins in cell signalling cascades and in particular, modulate the expression or activity of relevant enzymes, including phase 1 and phase 2 biotransformation enzymes. For example, flavonoids were shown to induce protective gene expression through the electrophile-responsive element (EpRE), a regulatory sequence involved in the co-ordinated transcriptional activation of genes associated with phase 2 biotransformation, such as NQO1, glutathione S-transferases and UDP-glucuronosyltransferase (Valerio *et al.* 2001; Boerboom *et al.* 2006; Lee-Hilz *et al.* 2006; Yueh and Tukey 2007). The selective induction of detoxifying enzymes, such as NQO1 represents one of the main strategies for chemoprotection and offers a promising way to reduce the risk of cancer and other chronic diseases (Fahey *et al.* 2004).

However, flavonoids were also reported to act as natural ligands for the aryl hydrocarbon receptor (AhR) (Ashida *et al.* 2000; Tutel'yan *et al.* 2003). The AhR is a ligand-dependent transcription factor acting through the xenobiotic-responsive element (XRE), a specific DNA sequence in the regulatory regions of certain phase 1 enzymes, like CYP1A1, involved in xenobiotic metabolism (Denison *et al.* 1988). Beside phase 1 enzymes, AhR also regulates the transcription of NQO1 and, for example, glutathione S-transferase P1, which both have, in addition to an EpRE, a functional XRE in their regulatory region (Jaiswal 1991; Nebert and Duffy 1997; Ma *et al.* 2004). It has been proposed that induction of EpRE-mediated gene expression by so-called bifunctional inducers actually occurs in two steps, the first one involving induction of phase 1 enzyme gene expression through XRE elements in the phase 1 gene regulatory region, which would result in the generation of the actual inducer of EpRE-controlled gene transcription in the second step (Miao *et al.* 2004). This order of consecutive molecular events suggests a direct relation between AhR and EpRE and places the EpRE-pathway downstream of XRE-mediated gene expression in the case of a bifunctional inducer (Miao *et al.* 2005). On the basis of their ability to induce phase 1 and/or phase 2 enzymes, inducers are therefore differentiated into two families: monofunctional inducers that act directly through the EpRE and selectively elevate phase 2 enzymes and bifunctional inducers that are able to induce both XRE- and EpRE-controlled gene expression and upregulate both phase 1 and phase 2 enzymes (Prochaska and Talalay 1988; Kohle and Bock 2006). Since phase 1 enzymes can activate procarcinogens and convert them to their ultimate reactive species, monofunctional agents that induce phase 2 enzymes selectively would be more desirable candidates for cancer chemoprevention. Although flavonoids have attracted considerable attention as phase 2 enzyme inducers (Yannai *et al.* 1998; Myhrstad *et al.* 2002; Lee-Hilz *et al.* 2006), several flavonoids are also reported to induce phase 1

enzymes (Canivec Lavier *et al.* 1996; Ciolino *et al.* 1999), and are therefore classified as bifunctional inducers (Yannai *et al.* 1998; Fahey and Stephenson 2002). So far, there is only little and conflicting experimental data available concerning the involvement of the AhR in induction of NQO1 gene expression by flavonoids (Yannai *et al.* 1998; Fahey and Stephenson 2002). These data do not allow to distinguish between a direct effect through the AhR and the XRE within the NQO1 regulatory region and, on the other hand, true bifunctional induction through XRE-mediated activation of the expression of an upstream gene generating the ultimate inducer acting through the NQO1 EpRE. Therefore it remains to be elucidated whether the induction of EpRE-mediated gene transcription by bifunctional flavonoids indeed requires an upstream induction of XRE-mediated gene expression.

The objective of this study is to elucidate whether flavonoid-induced EpRE-mediated gene transcription is dependent on preceding induction of XRE-mediated gene transcription. With the use of the same Hepa-1c1c7 cell line stably transfected with a firefly luciferase reporter gene under expression regulation of an EpRE from the human NQO1 gene (EpRE-LUX cells) or an XRE-containing sequence from the mouse cytochrome P4501A1 gene, it was possible to determine the relative gene induction response generated by flavonoids through each of these enhancer elements.

It has been reported that the structural properties of flavonoids play an important role in their ability to act as phase 1 or phase 2 enzyme inducer. For the activation of EpRE-mediated gene expression, the double bond between C2 and C3 and the hydroxylation of C3 of the flavonoid seem to play an important role (Lee-Hilz *et al.* 2006). Hydroxylation of the B-ring can contribute to the potential of a flavonoid to induce XRE-mediated gene expression, but the hydroxyl groups in the A-ring have negligible effect (Amakura *et al.* 2003). To test whether these structural properties influences the ability of flavonoids to act as mono- or bifunctional inducers, 5 flavonoid model compounds were chosen, which within the series differ by a systematic increase in the number of hydroxyl substituents (Fig. 4.1).

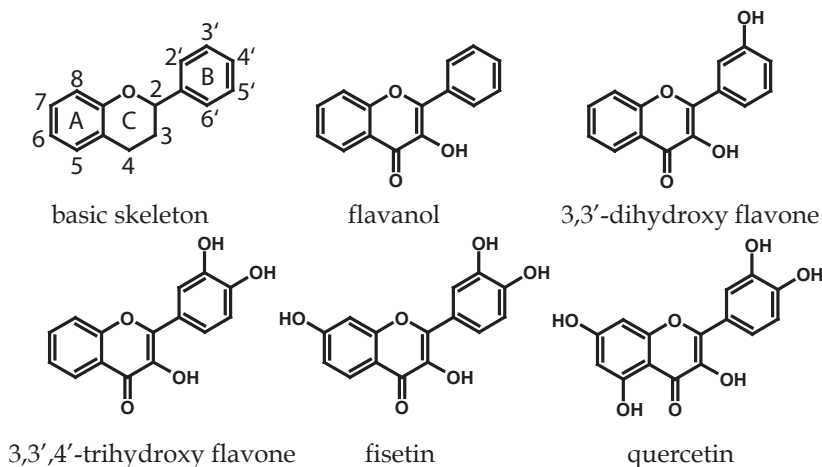


Figure 4.1: Chemical structure of the flavonoids tested in this study

This study shows that all tested flavonoids are able to induce the EpRE- and the XRE-mediated gene expression in Hepa-1c1c7 cells, but in a different concentration range, indicating that at physiological concentrations, only the induction of detoxifying enzymes via the EpRE-mediated pathway is relevant. The EpRE-mediated gene expression induced by flavonoids in Hepa-1c1c7 cells therefore appears not a downstream reaction of XRE-mediated gene expression.

4

Materials & Methods

Chemicals

Alpha-Modified Eagle's Medium (α -MEM), Hanks' balanced salt solution (HBSS), trypsin, foetal calf serum (FCS), phosphate-buffered saline (PBS), gentamicin and Geneticin (G418) were purchased from Gibco Invitrogen Corporation (Paisley, Scotland). Dimethyl sulphoxide (DMSO) was obtained from Acros Organics (New Jersey, USA). Flavanol, 3,3'-dihydroxyflavone, 3,3',4'-trihydroxyflavone, fisetin and quercetin were purchased from Extrasynthese (Genay Cedex, France). For all standards and substrates a fresh stock solution in DMSO was prepared for each experiment.

Cell lines

Hepa-1c1c7 mouse liver hepatoma cells were a kind gift from Dr. M.S. Denison, (University of California, Davis) and were stably transfected with the reporter vector pTI(hNQO1-EpRE)Luc⁺ carrying the EpRE from the human NQO1 gene regulatory region between -470 to -448 from the transcription initiation site (5'-AGT CAC AGT GAC TCA GCA GAA TC-'3) coupled to a luciferase reporter gene, resulting in the EpRE(hNQO1)-LUX cell lines as described elsewhere (Boerboom *et al.* 2006), and referred

to as EpRE-LUX in this paper. Furthermore, Hepa-1c1c7 cells were stably transfected with the luciferase reporter vector pGudLuc1.1, containing the mouse mammary tumor virus promoter under xenobiotic-responsive element-mediated control, resulting in the H1L1.1c7 cell line as described elsewhere (Aarts *et al.* 1995). Analogous to the EpRE-LUX cells, this cell line is referred to as XRE-LUX in this paper.

Both transfected Hepa-1c1c7 cell lines were cultured in α -MEM, supplemented with 10% FCS and 50 μ g/mL gentamicin and in addition 0.5 mg/mL G418 to maintain selection pressure on the presence of the reporter gene insertion. The cells were maintained in a humidified atmosphere with 5% CO₂ at 37°C. These transfected Hepa-1c1c7 cells, containing the luciferase gene under expression regulation of the EpRE and the XRE of human NQO1 will be further addressed as EpRE-LUX cells and XRE-LUX cells, respectively.

EpRE-LUX and XRE-LUX assay.

EpRE-mediated induction of gene expression in EpRE-LUX cells by flavonoids was tested using the EpRE-LUX luciferase reporter gene assay as described previously (Boerboom *et al.* 2006). The experimental procedure of the reporter gene assay for XRE-mediated gene induction (Aarts *et al.* 1995) was essentially similar. Briefly, EpRE- and XRE-LUX cells were cultivated as described above. To investigate the effect of inducers of EpRE- and XRE-mediated gene expression, suspensions of EpRE-LUX or XRE-LUX cells (2×10^5 cells/mL) were plated in culture medium in 96-wells view-plates (Corning, 100 μ L/well) and incubated for 24 hours to allow attachment of the cells to the bottom of the wells and the formation of a confluent monolayer. Next, the culture medium was removed and cells were treated with 200 μ L medium containing the flavonoid of interest. The DMSO concentration in the culture medium was kept constant at 0.5%. After 24 hours of exposure the cell layer was washed with 0.5 x PBS, and lysed by addition of Low Salt Buffer (10 mM Tris, 2 mM DTT and 2 mM trans-1,2-diaminocyclohexane-N,N,N',N'-tetra-acetic acid monohydrate; pH 7.8) followed by one freezing and thawing cycle. Luciferase reagent (20 mM Tricine, 1.07 mM (MgCO₃)₄Mg(OH)₂, 2.67 mM MgSO₄, 0.1 mM EDTA, 2 mM DTT, 0.47 mM D-luciferin, 5 mM ATP; pH 7.8) was injected and luciferase activity was immediately measured using a Luminoskan RS (Labsystems) luminometer. The luciferase expression measured was expressed as the induction factor (IF) defined as the potency of each flavonoid to increase the luciferase expression as compared to cells incubated with medium containing only 0.5% DMSO. Statistical significance of the response as compared to the solvent control was tested using Student's t-test (significant with $p < 0.05$).

Results

The flavonoids tested in this study were chosen on the basis of their related chemical structure and are depicted in Fig 4.1. To study the possible effects of hydroxylations on the XRE-mediated gene expression, as shown earlier for EpRE-mediated gene expression (Lee-Hilz *et al.* 2006), each flavonoid in the series differs in one hydroxylation position from its closest analogue (Fig. 4.1). The concentrations tested ranged between 0.1 μM to 100 μM . As exemplified for quercetin in Fig. 4.2, all flavonoids tested stimulated both EpRE- and also XRE-mediated luciferase induction in a concentration-dependent manner.

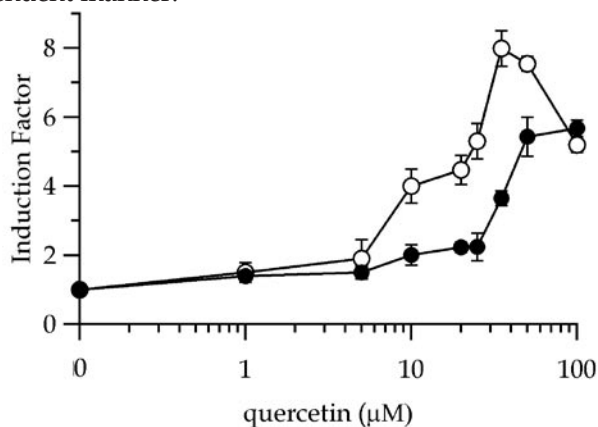


Figure 4.2: Induction of EpRE- (○) and XRE- (●) mediated gene transcription by quercetin. Data are presented as means with standard error based on six independent measurements. The response in both EpRE-LUX and XRE-LUX cells was found to be statistically significant ($p < 0.05$) over the entire concentration range tested (1–100 μM).

Quercetin induced EpRE-mediated gene expression already at relatively low concentrations, while XRE-mediated gene expression occurred at higher concentrations (Fig. 4.2). The maximal induction factors (IF) of EpRE- and XRE-mediated gene expression are shown in Table 4.1 for all flavonoids tested.

Table 4.1: Maximal induction factors (IF) and EC₅₀ values of flavonoids based on EpRE-LUX and XRE-LUX assay

| Inducer | Maximal induction factor (IF) | | EC ₅₀ (μM) | |
|---------------------------|-------------------------------|-----|-----------------------|-----|
| | EpRE | XRE | EpRE | XRE |
| Flavonol | 5.2 | 1.3 | 6 | nd |
| 3,3'-dihydroxyflavone | 5.8 | 7.9 | 3 | 15 |
| 3,3',4'-trihydroxyflavone | 6.2 | 2.0 | 5 | nd |
| fisetin | 8.0 | 6.4 | 4 | 33 |
| quercetin | 8.1 | 5.5 | 13 | 43 |

(nd means not detectable due to lack of sufficient induction)

As shown before (Lee-Hilz *et al.* 2006), flavonoids with a hydroxylation at the 3 position (Fig. 4.1) are good inducers of EpRE-mediated gene expression. Furthermore, the degree of hydroxylation also affects the inducing ability of the tested flavonoids, with quercetin being the most extensively hydroxylated and most potent inducer (maximal IF 8.1). However, no relationship between the position or the degree of hydroxylation and the ability of the tested flavonoids to induce XRE-mediated gene expression was observed and 3,3'-dihydroxyflavone showed to be the most potent inducer of XRE-mediated gene expression (IF 7.9) (Table 4.1). Therefore, it can be concluded that the degree of hydroxylation is not important for XRE-mediated gene expression.

The results show that all tested flavonoids are able to induce EpRE- as well as XRE-mediated gene expression, confirming that flavonoids comply with the current definition of bifunctional inducers (Table 4.1). However, except for 3,3'-dihydroxyflavone, all tested flavones were found to induce EpRE-mediated gene expression up to a higher factor than XRE-mediated gene expression.

Furthermore, the EC₅₀ values for each tested flavonoid were also determined (Table 4.1). Comparison of the results of the EC₅₀ of both cell lines indicates that transcription activation through the XRE occurs at substantially higher flavonoid concentrations than through the EpRE. For flavonol and 3,3',4'-trihydroxyflavone, the EC₅₀ could not be calculated, due to very low induction in the tested concentration range, with maximum induction factors of 1.3 and 2.0 fold for XRE-mediated gene expression, respectively. Higher concentrations of flavonol and 3,3',4'-trihydroxyflavone could not be tested due to cytotoxicity problems. Nevertheless, the results show that the induction of XRE-mediated gene expression by flavonol and 3,3',4'-trihydroxyflavone also starts to increase at a higher concentration level than the EpRE-mediated gene expression (Table 4.1).

For comparison, the EpRE- and XRE-mediated gene expression level attained in the physiologically relevant concentration range are compiled in Fig. 4.3.

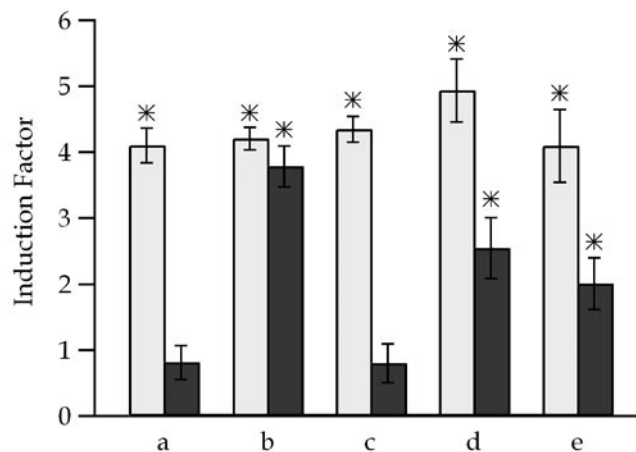


Figure 4.3: Induction factors (IF) of the tested flavonoids (a: flavonol, b: 3,3'-dihydroxyflavone, c: 3,3',4'-dihydroxyflavone, d: fisetin, e: quercetin) measured in the EpRE-LUX (white) and XRE-LUX (black) assay at the physiologically relevant concentration of 10 μ M. (*) indicates a response significantly different from the blank ($p < 0.05$).

At a physiological relevant flavonoid concentration of 10 μ M, significant EpRE-mediated gene expression can be clearly observed (Fig. 4.3). All tested flavonoids induce EpRE-mediated gene expression more than 4 fold, while significant XRE-mediated gene expression is only observed with 3,3'-dihydroxyflavone, fisetin and quercetin with induction factors of 3.7, 2.5 and 2.0 respectively (Fig. 4.3). XRE-mediated gene expression is not induced at this concentration by flavonol and 3,3',4'-dihydroxyflavone. Thus, with the exception of 3,3'-dihydroxyflavone, at a concentration of 10 μ M, the potency of flavonoids to induce EpRE-mediated gene expression is much more prominent than that for XRE-mediated gene expression.

Discussion

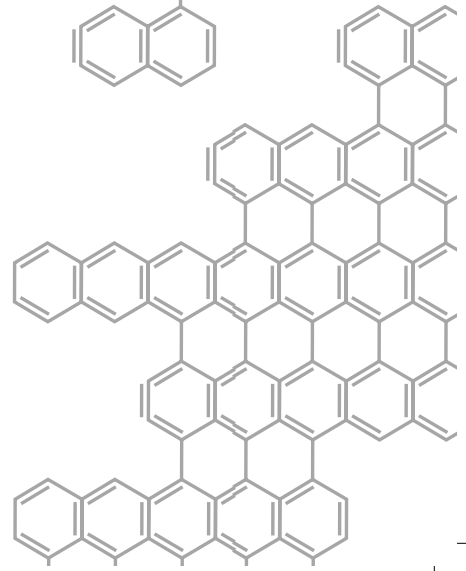
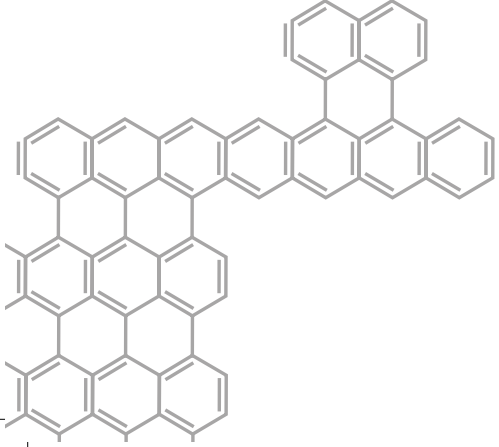
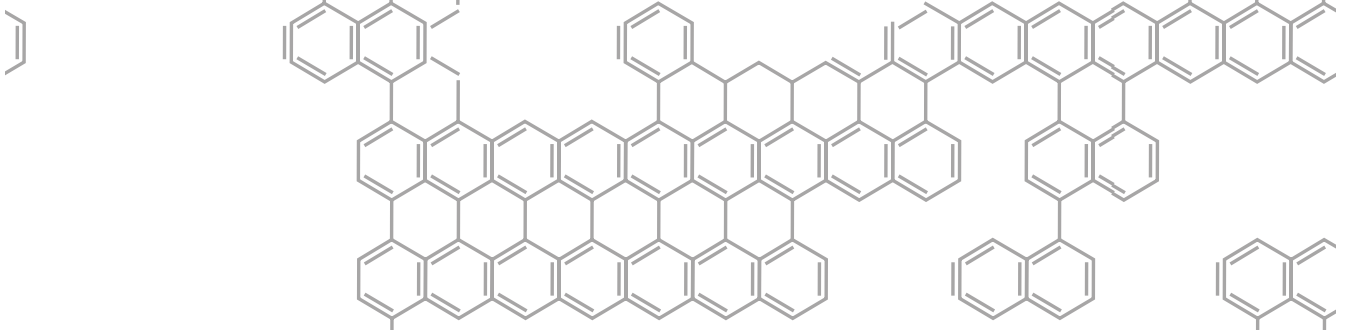
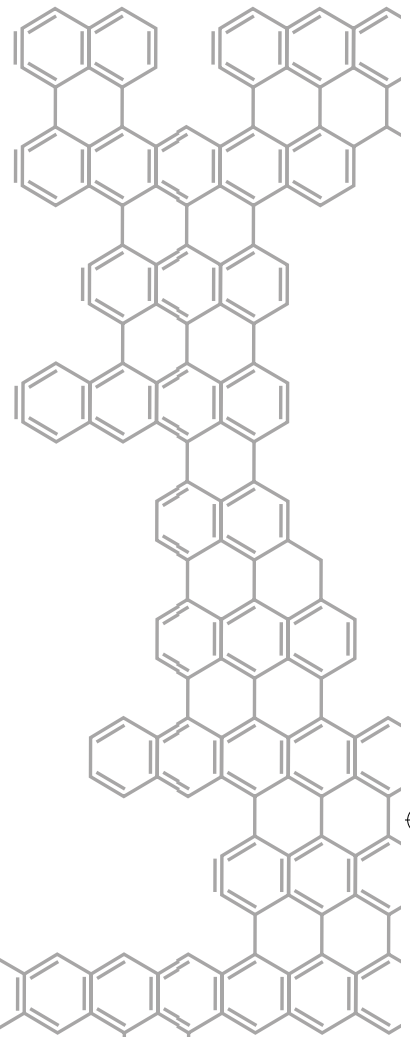
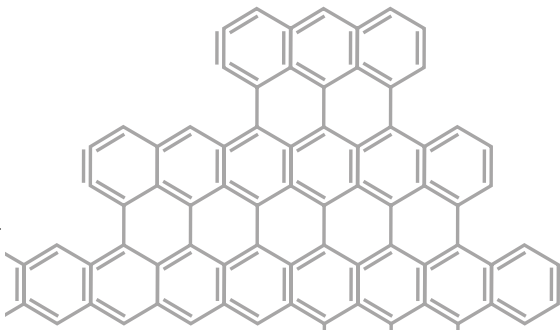
Flavonoids are bioactive food components, reported to exert protective effects against a variety of diseases, in particular various types of cancer (Fresco *et al.* 2006). The signal transduction pathways activated by flavonoids and their interactions have only been partially elucidated. Flavonoids are reported to induce EpRE-mediated transcription of detoxifying enzyme genes involved in chemoprotection (Boerboom *et al.* 2006; Lee-Hilz *et al.* 2006). Certain flavonoids were reported to be bifunctional inducers, implying AhR agonist activity, resulting in phase 1 enzyme gene induction besides being involved in activation of phase 2 enzymes via the EpRE (Amakura *et al.* 2003). To investigate whether upstream induction of XRE-mediated gene expression could play a role in the induction of EpRE-mediated gene transcription by bifunctional flavonoids, we tested several flavonoids on their ability to induce EpRE- as well as XRE-mediated gene expression in the same cellular background.

The results of this study show that all tested flavonoids are able to activate EpRE- as well as XRE-mediated gene expression, indicating that flavonoids are indeed bifunctional inducers according to the generally accepted definition (Table 4.1). β -Naphthoflavone was reported earlier to induce both XRE- and EpRE-mediated gene expression to a similar extent of 2.4 and 2.9 times, respectively (Rushmore and Pickett 1990). In contrast to this prototypical bifunctional inducer, most flavonoids tested in this study induced EpRE-mediated gene expression at relatively lower concentration, while relatively higher flavonoid concentrations were needed to induce XRE-mediated gene expression (Table 4.1). These results suggest that flavonoid-induced EpRE-mediated gene expression is unlikely to depend on major XRE-mediated gene expression. Although significant induction of XRE-mediated gene expression was observed at 10 μ M fisetin (IF 2.5) and quercetin (IF 2), it is clear, that also with these flavonoids the potency to induce EpRE-mediated gene expression (fisetin IF 5.0, quercetin IF 4.0) is much more prominent. Furthermore, with these flavonoids, the dose-response relation is much steeper (Fig. 4.2) and the EC₅₀ is lower for induction of EpRE-mediated gene expression (Table 4.1). This supports that XRE-mediated gene expression is not required for EpRE-mediated transcription activation by quercetin and fisetin. The only possible exception observed is 3,3'-dihydroxyflavone, which induced XRE-mediated (IF 3.8) and EpRE-mediated gene expression (IF 4.2) to a similar extent at 10 μ M concentration.

It is reported earlier, that at low concentrations, various flavonoids can act as AhR antagonist towards a strong agonist such as TCDD, by competing for the binding to the receptor (Ashida *et al.* 2000). In contrast, high concentrations of flavonoids may act as AhR agonists and potentiate the effects of TCDD, including transactivation of CYP1A1 genes (Lu *et al.* 1996). This is in line with our findings, showing that high flavonoid concentrations are generally needed to observe significant induction of XRE-mediated gene expression.

Consistent with our observations, prenylated chalcones and flavanones, considered as bifunctional inducers, were also reported to induce NQO1 activity but not CYP1A1 expression in Hepa1-c1c7 cells (Miranda *et al.* 2000). Furthermore, kaempferol, a strong inducer of EpRE-mediated gene expression was shown to be a natural ligand of AhR but failed to affect CYP1A1 expression. These findings are thus in line with our observations that at physiological relevant concentrations of flavonoids, induction of EpRE-mediated gene expression is generally more prominent than induction of XRE-mediated gene expression. However, it cannot be completely ruled out on the basis of this study, that gene expression modulation by flavonoids might involve a functional interaction between the AhR/XRE and the Nrf2/EpRE gene regulatory pathways, either involving the background levels or minor induction of active AhR (Ma *et al.* 2004). In spite of the fact that flavonoids are omnipresent in our diet, the concentrations achieved *in vivo* following dietary administration tend to be low due to the low oral bioavailability of many flavonoids. Reported concentrations of quercetin (free and conjugated) in human serum and plasma can reach levels up to 10 μM after dietary supplementation at non-toxic levels (Hollman *et al.* 1996; Olthof *et al.* 2000; Walle *et al.* 2001). Therefore, at concentrations above 10 μM , which is essentially the concentration range in which XRE-mediated gene expression only starts to be induced effectively (Fig. 4.2 and 4.3), the availability of flavonoids is questionable.

Taken together, the results of this study show that flavonoids, although they are able to induce both XRE- and EpRE-controlled gene expression, and therefore are classified as bifunctional inducers, preferentially induce EpRE-mediated gene transcription in mouse hepatoma cells at physiologically relevant concentrations. This leads to the conclusion that the current definition of a bifunctional inducer should be refined and take into account that both the XRE- and the EpRE-mediated induction functions should in principle be operational at equivalent concentrations and simultaneously in target cells. Therefore, comprehensive analysis of the possible cross-talk between the AhR/XRE and the Nrf2/EpRE gene regulatory pathways requires complementary studies of bifunctional inducers throughout their entire physiological concentration range.





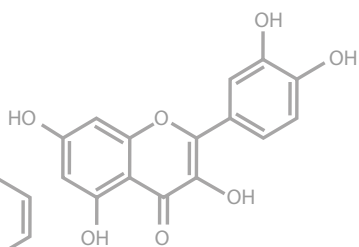
Chapter 5

Pro-oxidant activity of flavonoids induces EpRE-mediated gene expression

Yee Y. Lee-Hilz, Anne-Marie J.F. Boerboom, Adrie H. Westphal, Willem J. H. van Berkel, Jac M. M. J. G. Aarts and Ivonne M. C. M. Rietjens



Chemical Research in Toxicology 19 (2006) 1499-1505



Abstract

Flavonoids are important bioactive dietary compounds. They induce electrophile-responsive element (EpRE)-mediated expression of enzymes, such as NAD(P)H:quinone oxidoreductase 1 (NQO1) and glutathione S-transferases (GST), which are major defense enzymes against electrophilic toxicants and oxidative stress. Induction of EpRE-mediated gene transcription involves the release of the transcription factor Nrf2 from a complex with Keap1, either by a direct interaction of the inducer with Keap1, or by protein kinase C (PKC)-mediated phosphorylation of Nrf2. Inhibition of PKC in Hepa-1c1c7 cells, stably transfected with human NQO1-EpRE-controlled luciferase revealed that PKC is not involved in flavonoid-induced EpRE-mediated gene transcription. However, the ability of flavonoids to activate an EpRE-mediated response correlates with their redox properties characterized by quantum mechanical calculations. Flavonoids with a higher intrinsic potential to generate oxidative stress and redoxcycling, are the most potent inducers of EpRE-mediated gene expression. Modulation of the intracellular glutathione (GSH) level showed that the EpRE-activation by flavonoids increased with decreasing GSH and vice versa, supporting an oxidative mechanism. In conclusion, the pro-oxidant activity of flavonoids can contribute to their health promoting activity by inducing important detoxifying enzymes, pointing at a beneficial effect of a supposed toxic chemical reaction.

Introduction

Fruit- and vegetable-rich diets are associated with reduced incidence of various cancer types, and flavonoids are important key compounds in these food items, considered to be health-protecting (Manach *et al.* 2004). The estimated daily intake of flavonoids ranges up to 1 g/day (Scalbert and Williamson 2000). Flavonoids have been reported to protect against coronary heart disease, stroke and certain cancer types through their antioxidant, anti-inflammatory, anti-allergic and antiviral activities (Scalbert *et al.* 2005). However, also pro-oxidant activity of flavonoids has been reported (Awad *et al.* 2001; Galati and O'Brien 2004). The cancer-preventive activity of flavonoids has been attributed to multiple parallel mechanisms. One important mechanism is the induction of detoxifying enzymes by flavonoids, such as glutathione S-transferases, UDP-glucuronosyltransferases, γ -glutamylcysteine synthetase, NAD(P)H: quinone oxidoreductase 1, heme oxygenase-1, epoxide hydrolase, leukotriene B4 dehydrogenase and aldehyde dehydrogenase (Chen and Kong 2004; Lee *et al.* 2005). These enzymes play a central role in the defense system of cells, being able to detoxify reactive genotoxic substances and to contribute significantly to the cellular protection against redox cycling and oxidative stress (Chen and Kong 2004).

Regulation of this protective gene expression by dietary chemopreventive compounds can be mediated by the electrophile-responsive element (EpRE), initially referred to as the antioxidant-responsive element (ARE) (Prester and Talalay 1995). The EpRE is a regulatory sequence involved in the coordinated transcriptional activation of genes associated with phase 2 biotransformation, protection against oxidative stress and other cancer-chemoprotective mechanisms (Dinkova-Kostova *et al.* 2005a). The key regulator of EpRE-mediated gene expression is the transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2) and, to a lesser extent, Nrf1, which both are members of the nuclear basic leucine zipper transcription factors (Jaiswal 2004). The major regulator of Nrf2 is identified to be Keap1 (Kelch-like erythroid cell-derived protein with CNC homology-associating protein 1), a dimeric cytoplasmic actin-binding protein (Kang *et al.* 2004), which represses Nrf2 transcription activation by cytoplasmic sequestration and mediating degradation of Nrf2 (Itoh *et al.* 1999). Several mechanisms of Nrf2 activation resulting in the release of Nrf2 from Keap1 have been proposed. A suggested pathway of increased EpRE-mediated gene induction by nuclear Nrf2 accumulation is through phosphorylation of Nrf2 by protein kinase C (PKC) leading to the dissociation of Nrf2 from the complex (Huang *et al.* 2002; Bloom and Jaiswal 2003; Numazawa *et al.* 2003).

Another proposed mechanism is the direct reaction of oxidative compounds with the Keap1-Nrf2 complex (Zhang and Hannink 2003). The fact that the dimeric Keap1 contains multiple cysteine residues in each monomer, many of which are potential sites of oxidative attack by inducers of the EpRE-mediated gene expression, has led to the suggestion that the Keap1-Nrf2 interaction constitutes a sensor of oxidative stress involved in triggering EpRE-controlled responses to restore the physiological redox

status in cells (Zhang and Hannink 2003; Kobayashi *et al.* 2004; Dinkova-Kostova *et al.* 2005b). The release of Nrf2 from Keap1, leading to activation of EpRE-mediated gene transcription, is reported to be a redox-dependent process (Sekhar *et al.* 2002) and activated by ROS and/or electrophiles (Dinkova-Kostova *et al.* 2005b; Velichkova and Hasson 2005). It is suggested that these inducers can interact with reactive thiol groups of the Keap1 protomers, resulting in intermolecular disulfide formation and conformational changes ultimately resulting in Nrf2 release (Dinkova-Kostova *et al.* 2005a; Dinkova-Kostova *et al.* 2005b).

The molecular mechanism by which flavonoids are able to induce detoxifying enzymes is not yet known. Although it was shown that flavonoids induce detoxifying enzymes via an EpRE-mediated response (Valerio *et al.* 2001; Myhrstad *et al.* 2002; Chen and Kong 2004; Boerboom *et al.* 2006), flavonoids as such do not have electrophilic activity, but are commonly known to have electron donating antioxidant properties (Williams *et al.* 2004). However, we recently showed that flavonoid metabolites do have electrophilic activity and can covalently bind to GSH and DNA (van der Woude *et al.* 2006). Therefore, the objective of this study is to elucidate by which mechanism flavonoids are able to induce EpRE-mediated induction of detoxifying enzymes.

Therefore, we investigated the EpRE-mediated gene expression induced by a series of flavonoids in Hepa-1c1c7 cells, stably transfected with a luciferase reporter gene under the control of the EpRE derived from the human *NQO1* gene (EpRE-LUX cells). The induction potential of flavonoids in EpRE-LUX cells was studied in the presence of the PKC inhibitor staurosporine as well as correlated with the redox properties of the inducers as quantified by molecular orbital calculations. In addition, the induction potential of flavonoids was studied on EpRE-LUX cells with modified intracellular GSH levels. The results obtained indicate a role for flavonoid pro-oxidant chemistry in their mechanism of EpRE-mediated gene expression control.

Materials & Methods

Materials

Alpha-Modified Eagle's Medium, Hanks' balanced salt solution (HBSS), trypsin, foetal calf serum (FCS), phosphate-buffered saline (PBS), gentamicin and G418 were purchased from Gibco Invitrogen Corporation (Breda, The Netherlands). Dimethyl sulfoxide (DMSO) was obtained from Acros Organics (New Jersey, USA). All tested flavonoids were purchased from Extrasynthese (Genay Cedex, France). For each experiment a fresh stock solution in DMSO of all standards and substrates was prepared.

Cell lines

Hepa-1c1c7 mouse hepatoma cells were a kind gift from Dr. M.S. Denison, (University of California, Davis) and were cultured in alpha-Modified Eagle's Medium, supplemented with 10% FCS and 50 µg/mL gentamicin. The cells were maintained in a humidified atmosphere with 5% CO₂ at 37°C. Hepa-1c1c7 cells were stably transfected with the reporter vector pTI(hNQO1-EpRE)Luc⁺ carrying the EpRE from the human NQO1 gene regulatory region between -470 to -448 (5'-AGT CAC AGT GAC TCA GCA GAA TC-3') coupled to a luciferase reporter gene, as described previously (Boerboom *et al.* 2006). The culture medium of the transfected Hepa-1c1c7 cells was the same as for the wild-type Hepa-1c1c7 cells, containing in addition 0.5 mg/mL G418. These transfected Hepa-1c1c7 cells, containing the luciferase gene under expression regulation of the EpRE from the human NQO1 gene will further be addressed to as EpRE-LUX cells.

EpRE-LUX assay

EpRE-mediated induction of gene expression by flavonoids was tested using the EpRE-LUX luciferase reporter gene assay as described previously (Boerboom *et al.* 2006). Briefly, EpRE-LUX cells were cultivated as described above. To investigate the effect of inducers of EpRE-mediated gene expression, cell suspensions (2x10⁵ cells/mL) were plated in culture medium in 96-wells view-plates (Corning, 100 µL/well) and incubated for 24 hours to allow attachment of the cells to the bottom of the wells and the formation of a confluent monolayer. Next, the culture medium was removed and cells were treated with 200 µL medium containing the flavonoid of interest. The DMSO concentration in the culture medium was kept constant at 0.5%. After 24 hours of exposure cells were washed with 0.5 x PBS, harvested and homogenized in Low Salt Buffer (10 mM Tris, 2 mM DTT and 2 mM trans-1, 2-diaminocyclohexane-N,N,N',N'-tetra-acetic acid mono-hydrate; pH7.8). Luciferase reagent (20 mM tricine, 1.07 mM (MgCO₃)₄Mg(OH)₂, 2.67 mM MgSO₄, 0.1 mM EDTA, 2 mM DTT, 0.47 mM D-luciferin, 5 mM ATP; pH7.8) was added and luciferase activity was measured using a Luminoskan RS (Labsystems) luminometer.

The role of PKC in the induction of EpRE-mediated gene expression by flavonoids

The effect of PKC inhibition on EpRE-mediated luciferase induction by flavonoids was investigated using the PKC inhibitor staurosporine. EpRE-LUX cells were cultured as described above and cell suspensions (2x10⁵ cells/mL) were plated in culture medium in 96-wells view-plates (Corning, 100 µL/well) and incubated for 24 hours to allow formation of a confluent monolayer. Next, culture medium was removed and cells were treated with staurosporine concentrations ranging from 0.5 nM - 10 nM in culture medium without FCS supplementation. After 3 hours pre-treatment of the cells with staurosporine, medium was removed and 200 µL medium without

FCS supplementation, containing the same amount of staurosporine, and in addition the inducer of EpRE-mediated gene expression to be tested was added to the cells. The DMSO concentration in the culture medium was kept constant at 0.5%. After 24 hours of exposure cells were washed with 0.5 x PBS, harvested and homogenized in Low Salt Buffer. Luciferase reagent was added and luciferase activity was measured as described above.

Quantum mechanical calculations

The quantum mechanical calculations were carried out with Spartan04 for Windows® Version 1.0.3 (Wavefun, CA, USA). All possible geometrical conformers of each flavonoid were used as input for the semiempirical molecular orbital calculations Austin Model 1 (AM1) and the E_{HOMO} energy (eV) and the van der Waals volume (\AA^3) of the most probable conformer, the one with the lowest heat of formation, were chosen to correlate with the induction factor observed for EpRE-mediated gene expression.

Statistical analysis

The Statistical Package for Social Scientists (SPSS) 10.1 for Windows (SPSS, Chicago, IL, USA) was used to correlate the experimental data with the values derived from quantum mechanical calculations.

Cross-validation was performed using the leave-out-many method, with 20% of the calibration compounds left out at each step (Eriksson *et al.* 2003). To reduce bias, the validation groups were created using the method of unsupervised stratification and the data were ranked according to increasing EHOMO values. The internal cross-validated coefficient of determination (q^2) was calculated using:

$$q^2 = 1 - (\text{PRESS}/\text{SSD})^2 ,$$

where the predictive sum of squares (PRESS) is the sum of the squared differences between actual and predicted induction factor and SSD is the sum-of-squares deviation for each actual induction factor from the mean induction factor of all the compounds. The correlation is acceptable when $q^2 > 0.5$ and $r^2 - q^2 < 0.3$ (Eriksson *et al.* 2003), with r^2 being the correlation coefficient.

Effect of oxidative stress on EpRE-mediated gene induction by flavonoids

To monitor the ability of flavonoids to induce EpRE-mediated gene expression of phase 2 enzymes through their pro-oxidant properties, the intracellular GSH level was modulated by addition of N-acetyl-L-cysteine (NAC), a precursor of GSH able to generate high levels of GSH in cells (Qanungo *et al.* 2004), and by the addition of BSO to decrease the intracellular level of GSH (Hansen *et al.* 2004). Cells were cultured and plated out as described above. Culture medium was removed after 24 hours of

incubation and the cell monolayers were treated with different concentration of NAC in the medium ranging from 0.01 mM – 40 mM, or BSO in the medium ranging from 5 μ M – 100 μ M. After 4 hours pre-incubation with NAC or 24 hours with BSO, to allow increase or decrease in GSH inside the cells, medium was removed and 200 μ L medium containing NAC or BSO and the inducers of interest were added to the cells. The DMSO concentration in the culture medium was kept constant at 0.5%. After 24 hours of exposure cells were washed with 0.5 \times PBS, harvested and homogenized in Low Salt Buffer. Luciferase reagent was added and luciferase activity was measured as described above.

Cytotoxicity

Cytotoxicity of test compounds was determined using the lactate dehydrogenase (LDH) assay with minor adaptations for 96-well-plates (Mitchell and Acosta 1980). Briefly, cells were plated 24 hours before exposure at a density of 10^4 -cells per well in a 96-well-plate. Subsequently, for testing the cytotoxicity of the flavonoids, staurosporine, BSO or NAC, 200 μ L of culture medium containing different concentrations of the specific test substance was added. The DMSO concentration in the culture medium was kept constant at 0.5%. After 24 hours of exposure, culture medium was collected, cells were lysed and LDH activity was measured in the culture medium and in the cell lysate. Cytotoxicity was expressed as the ratio of extracellular to total LDH activity found inside and outside the cells. Under the experimental condition used, no cytotoxicity was observed with any of the tested compounds.

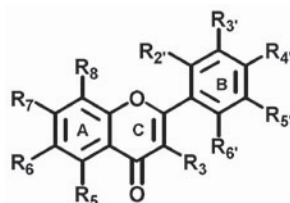
Results

Activation of EpRE-controlled gene expression by flavonoids

The chemical structures of the tested flavonoids are shown in Table 5.1.

Table 5.1: Structure, induction factor (IF) of EpRE-mediated gene transcription, calculated E_{HOMO} energy and Van der Waals volume (VdW) of each tested flavonoid

| Flavonoid | | | | | | |
|-----------|-----------------|---------------------------|------------------|-----|------------------------|-----------------------|
| | | OH | OCH ₃ | IF | E_{HOMO} (eV) | VdW (Å ³) |
| 1 | flavone | - | - | 2.8 | -9.27 | 232.75 |
| 2 | 5-OH flavone | R5 | - | 2.5 | -9.12 | 239.45 |
| 3 | 7-OH flavone | R7 | - | 2.5 | -9.36 | 240.12 |
| 4 | chrysin | R5, R7 | - | 2.1 | -9.25 | 246.61 |
| 5 | 3,7-OH flavone | R3, R7 | - | 3 | -8.89 | 246.87 |
| 6 | 6,7-OH flavone | R6, R7 | - | 3 | -9.10 | 247.21 |
| 7 | 3,3'-OH flavone | R3, R3' | - | 5.8 | -8.88 | 246.88 |
| 8 | 7,8-OH flavone | R7, R8 | - | 2.2 | -9.23 | 247.18 |
| 9 | galangin | R3, R5, R7 | - | 4.0 | -8.90 | 253.37 |
| 10 | resokaempferol | R3, R7, R4' | - | 6.5 | -8.72 | 254.06 |
| 11 | baicalein | R5, R6, R7 | - | 2.5 | -8.99 | 253.59 |
| 12 | apigenin | R5, R7, R4' | - | 2.0 | -9.15 | 253.80 |
| 13 | kaempferol | R3, R5, R7, R4' | - | 7.1 | -8.74 | 260.54 |
| 14 | luteolin | R5, R7, R3', R4' | - | 3.0 | -9.09 | 260.89 |
| 15 | fisetin | R3, R7, R3', R4' | - | 8.0 | -8.69 | 261.14 |
| 16 | quercetin | R3, R5, R7, R3', R4' | - | 10 | -8.72 | 267.65 |
| 17 | morin | R3, R5, R7, R2', R4' | - | 8.4 | -8.81 | 267.39 |
| 18 | myricetin | R3, R5, R7, R3', R4', R5' | - | 9 | -8.80 | 274.75 |
| 19 | tectochrysin | R5 | R7 | 4.4 | -9.16 | 266.76 |
| 20 | genkwanin | R5, R4' | R7 | 5 | -9.09 | 273.80 |
| 21 | isorhamnetin | R3, R5, R7, R4' | R3' | 7.8 | -8.65 | 287.80 |



To measure the potential of flavonoids to induce EpRE-mediated gene expression, Hepa-1c1c7 cells containing a firefly luciferase reporter gene under expression regulation of an EpRE from the human *NQO1* gene (EpRE-LUX cells) were used (Boerboom *et al.* 2006). The induction factor is defined as the potency of each flavonoid to increase the luciferase expression as compared to cells incubated with control medium only. All flavonoids tested showed, as shown for the examples quercetin, kaempferol, fisetin and apigenin (Fig. 5.1), a concentration-dependent luciferase induction.

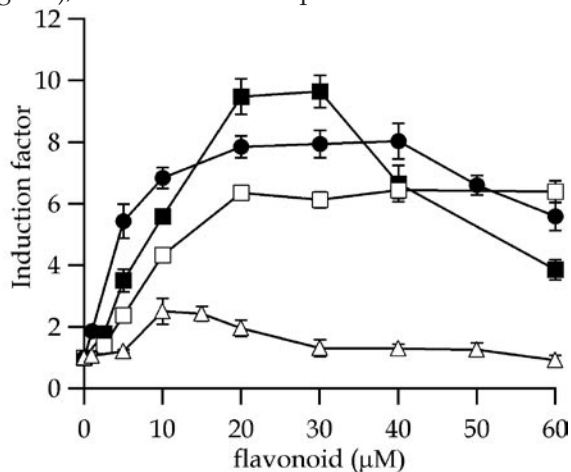


Figure 5.1: Induction of EpRE-mediated gene transcription by flavonoids. Effect of quercetin (-■-), fisetin (-▲-), kaempferol (-□-) and apigenin (-△-) on EpRE-mediated luciferase induction. Data are presented as means with standard error based on six independent measurements.

The concentrations tested ranged between 0.1 μM to 60 μM flavonoid. Table 5.1 shows the maximal level of induction (IF) observed for each flavonoid, which was reached at a concentration of 10 to 20 μM . Generally, flavonoids bearing a hydroxyl group at the 3-position are the best inducers of EpRE-mediated luciferase induction. Induction factors with these compounds ranged from 3-fold for 3,7-OH-flavone up to 10-fold for quercetin, while flavonoids without a hydroxyl group at the 3-position only show a low luciferase induction. In addition, three methylated flavonoid derivatives were included in this study. Isorhamnetin, the 3'-O-methylated metabolite of quercetin, shows a lower EpRE-mediated response of 7.8-fold, compared to quercetin with a 10-fold induction. In contrast, tectochrysin, the 7-O-methyl derivative of chrysin, with 4.4-fold induction and genkwanin, the 7-O-methyl derivative of apigenin, with an induction factor of 5-fold show a higher EpRE-mediated response compared to chrysin with 2.1-fold induction and apigenin with 2-fold induction. There is no significant correlation between luciferase induction and the degree of hydroxylation of flavonoids.

Involvement of PKC in EpRE-mediated gene transcription activation

To investigate if EpRE-mediated transcription activation by flavonoids requires PKC activity, luciferase induction in the EpRE-LUX reporter cells by tBHQ, a standard inducer of the EpRE-mediated gene transcription and two main dietary flavonoids with high inducing activity of EpRE-mediated gene expression, quercetin, and kaempferol, was studied in the presence of staurosporine, a standard inhibitor of PKC. Fig. 5.2A shows the effect of increasing staurosporine concentration on the tBHQ-mediated luciferase induction in EpRE-LUX cells.

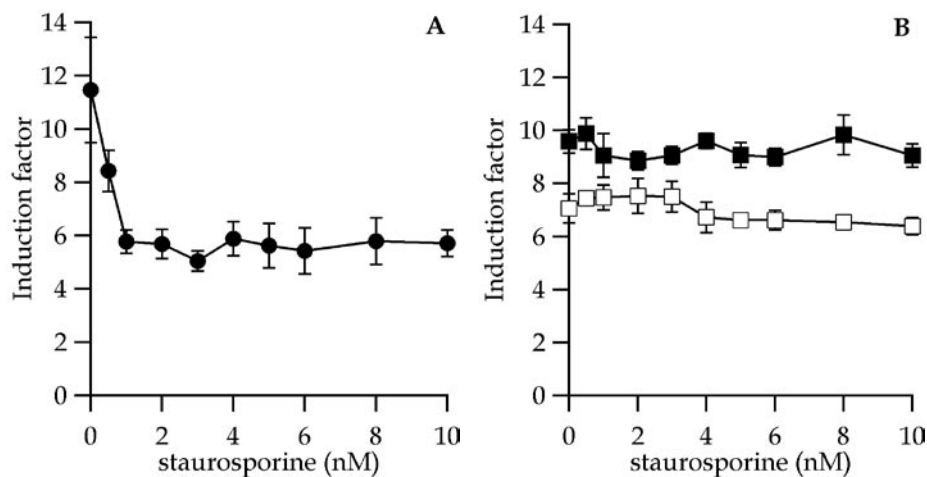


Figure 5.2: Effect of PKC inhibition by staurosporine on tBHQ- and flavonoid-induced EpRE-mediated gene transcription. **A** Effect of staurosporine on EpRE-mediated luciferase expression induced by 15 μ M tBHQ. The effect of staurosporine was found significant ($p < 0.05$) starting from 1 nM staurosporine (Student's *t*-test). **B** The effect of staurosporine on EpRE-mediated luciferase expression induced by 20 μ M of quercetin (-■-) or kaempferol (-□-), respectively. All data are presented as means with standard error based on six independent measurements

Significant inhibition of the luciferase induction by tBHQ was already visible at 1 nM staurosporine. The tBHQ-mediated luciferase induction decreased from 12- to 5.5-fold (60% reduction) and remained nearly constant between 1 nM and 10 nM staurosporine (Fig. 5.2A). Staurosporine showed no luciferase inducing activity by itself up to a concentration of 10 nM (data not shown).

In contrast to the inhibition of the luciferase induction response to tBHQ, the induction of luciferase by quercetin and kaempferol in EpRE-LUX cells was not inhibited by the PKC inhibitor staurosporine up to a concentration of 10 nM (Fig. 5.2B).

Correlation of the redox properties of flavonoids with their induction of EpRE-mediated gene expression.

The E_{HOMO} values of the tested flavonoids are presented in Table 5.1, listing the E_{HOMO} values of the conformer with the lowest heat of formation, representing the most probable conformation. Figure 5.3 shows the relation between the experimental induction factors (IF) of the EpRE-mediated gene transcription and the E_{HOMO} values of the tested flavonoids.

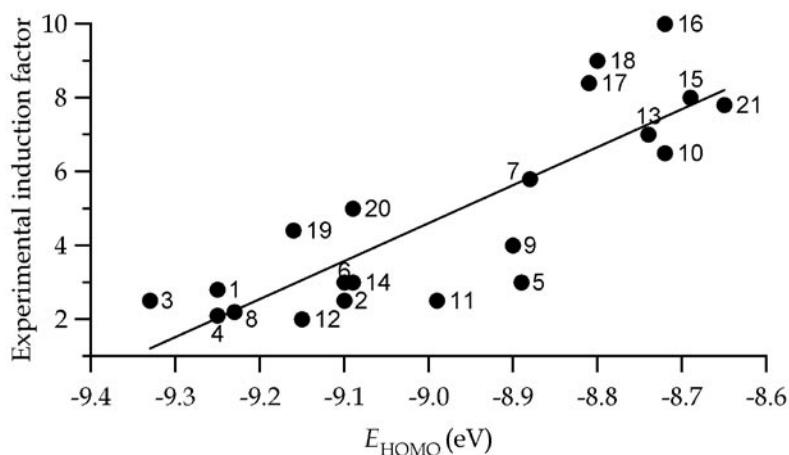


Figure 5.3: Correlation ($r^2=0.701$, $n=21$) as observed for the tested flavonoids between the induction factor for EpRE-mediated gene transcription and redox properties quantified by their E_{HOMO} (eV). The numbers correspond to the numbers in Table 5.1.

A linear correlation with $r^2 = 0.701$ is obtained ($n=21$). Because, steric parameters can be important for the interaction of inducers with the Keap1-Nrf2 complex, it was investigated whether the Van der Waals volumes (VdW) of the flavonoids would provide a suitable second descriptor for a quantitative structure activity relationship. The Van der Waals volumes of the flavonoids are presented in Table 5.1. Using these Van der Waals volumes, a two parameters quantitative structure activity relationship could be obtained (Equation 5.1):

$$\text{Predicted IF} = 7.83 \cdot E_{\text{HOMO}} + 0.059 \cdot \text{VdW} + 59.97$$

Figure 5.4 shows the relation between the experimental induction factors (IF) of the EpRE-mediated gene transcription and the induction factors predicted by Equation 5.1.

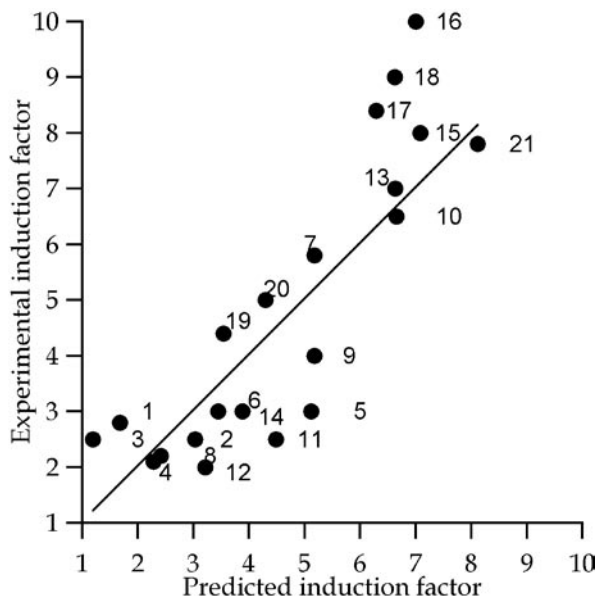


Figure 5.4: Correlation ($r^2=0.760$, $n=21$) between the observed EpRE-mediated gene transcription induced by flavonoids and the induction factors predicted by Equation 5.1 using E_{HOMO} and VdW as descriptors. The numbers correspond to the numbers in Table 5.1.

A linear correlation is obtained with $r^2 = 0.760$ ($n = 21$). The internal cross-validated coefficient of the correlation between the experimental induction factor and the predicted induction factor is $q^2 = 0.638$, showing the validity of this correlation. Together, these data indicate that the E_{HOMO} value of each flavonoid appears to be an important factor, with the Van der Waals volume being a minor determinant, for predicting its inducing capacity of EpRE-mediated response.

Role of the pro-oxidant activity of flavonoids in EpRE-mediated gene transcription activation

Three flavonoids with high EpRE-mediated response (quercetin, fisetin and kaempferol) were selected to investigate whether flavonoids induce EpRE-mediated gene transcription by generating oxidative stress. The luciferase induction mediated by these three flavonoids was investigated in EpRE-LUX cells in which the intracellular concentration of GSH, a major compound present in cells for protection against oxidative stress, was varied. Figure 5.5 shows the effect of modulation of the intracellular GSH concentration on the induction of EpRE-controlled luciferase expression as mediated by tBHQ, quercetin, fisetin and kaempferol.

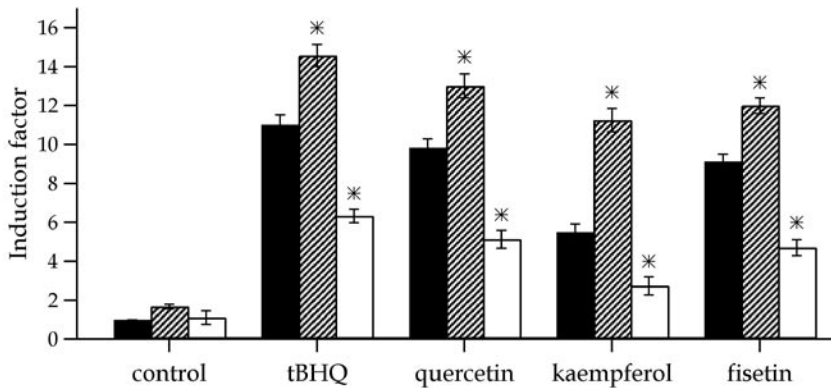


Figure 5.5: Effect of changes in intracellular GSH levels on tBHQ and flavonoid-induced EpRE activation. GSH levels were decreased by BSO and increased by NAC. Luciferase induction by 15 μ M tBHQ, 20 μ M quercetin, 20 μ M kaempferol, 30 μ M fisetin, and the control with only medium in EpRE(hNQO1)-LUX cells in absence (black bar) or presence of 100 μ M BSO (striped bar) or 40 mM NAC (white bar). All data are presented as means with standard error based on six independent measurements and (*) indicates a response significantly different from inducers alone ($p < 0.05$, Student's t-test).

Addition of 40 mM NAC, which increases GSH levels in cells (Qanungo *et al.* 2004), resulted in a significant decrease in luciferase induction with all tested compounds. The induction factor of tBHQ decreased from 11.0- to 6.3-fold (43% decrease, Fig. 5.5), of quercetin from 9.8- to 5.1-fold (48% decrease), of fisetin from 9.1-fold to 4.7-fold (48% decrease) and the kaempferol-mediated luciferase induction decreased from 5.5- to 2.7-fold (51% decrease) (Fig. 5.5). NAC showed no significant effect on luciferase expression in non-induced (control) EpRE-LUX cells up to a concentration of at least 40 mM (Fig. 5.5), indicating that an increase in intracellular GSH levels by itself does not affect the luciferase activity of EpRE-LUX cells.

Addition of BSO, which decreases the intracellular cytosolic GSH level (Hansen *et al.* 2004), resulted in an increase in the EpRE-controlled luciferase induction by the test compounds (Fig. 5.5). In the presence of 100 μ M BSO, tBHQ-, quercetin- and fisetin-mediated luciferase induction increased up to 14.6-fold (33% increase), 13-fold (33% increase) and 12-fold (33% increase) respectively. For kaempferol, a 2-fold increase in luciferase induction (5.5- to 11.3-fold, 105%) was observed. BSO alone also stimulated luciferase inductions by a factor of 1.6-fold in EpRE-LUX cells in a concentration dependent manner (Fig. 5.5). However, the response attained by flavonoid treatment in the presence of BSO is always considerably higher than the sum of the responses induced by BSO and flavonoid individually. This proves that the depletion of GSH with BSO leads to a more than additive enhancement of the EpRE-mediated gene expression activation by the inducers tested.

Discussion

Flavonoids are reported to induce phase 2 enzymes, which are important detoxifying enzymes in cells and suggested to play a role in the prevention against cancer (Chen and Kong 2004). The induction mechanism of detoxifying enzymes has been extensively studied, and activation of gene expression through the EpRE has been described for various flavonoids. Among these compounds, flavones are found to be the most potent inducers of the EpRE-mediated gene expression (Valerio *et al.* 2001; Myhrstad *et al.* 2002; Boerboom *et al.* 2006). Therefore, we tested 21 flavones, including some methylated derivatives on their ability to induce the EpRE-mediated gene expression. For this purpose our newly developed EpRE-LUX cell assay (Boerboom *et al.* 2006) was used, which provides a powerful tool to measure EpRE-mediated transcription activation.

The results of the flavonoid-induced EpRE-mediated gene transcription show that hydroxylation at the 3-position of the flavonoids is important for the EpRE-controlled gene induction (Table 1). This finding is in line with other studies of flavonoid-mediated induction of NQO1-activity (Uda *et al.* 1997). Furthermore, our findings suggest that the degree of hydroxylation of the flavonoids seems not to be important for the EpRE-mediated gene transcription (Table 5.1).

The bioavailability of flavones is relatively low. Plasma concentration of quercetin in humans can reach up to 7 μM (Manach *et al.* 2005). The concentrations tested in this study ranged from 0.5 to 60 μM . Although the maximal level of gene induction, which is reached with a concentration of 10 to 20 μM flavones, might not occur in normal physiological conditions, substantial induction is already found in the physiological concentration range (Fig. 5.1).

The release of Nrf2 from Keap1-Nrf2 complex is a crucial step in the EpRE-mediated gene induction of detoxifying enzymes and at least two important mechanisms for this event have been proposed. One proposed mechanism concerns an indirect effect of the inducer involving the activation of protein kinase C (PKC), resulting in release of Nrf2 from Keap1 through phosphorylation of Nrf2 (Bloom and Jaiswal 2003). Beside PKC, kinases like MAPK (mitogen-activated protein kinase) and PI3K (phosphatidylinositol 3-kinase) might also play a role in EpRE-mediated gene transcription (Yu *et al.* 1999; Kim and Kim 2004). To test the involvement of PKC in the EpRE-mediated gene transcription activation by flavonoids, we used staurosporine as a specific inhibitor of PKC to study inhibition of the luciferase induction response by tBHQ and flavonoids. Besides being a strong inhibitor of PKC (Tamaoki *et al.* 1986), staurosporine is reported to inhibit most protein kinases with IC_{50} values in the range of 1-20 nM (Howard-Jones and Walsh 2005). Therefore, the experiments of the present study also give an indication on the involvement of MAPK and PI3K in the flavonoid-mediated transcription of detoxifying enzymes. In line with other studies (Nguyen *et al.* 2003) the EpRE-mediated gene transcription by tBHQ, which is generally used as

a standard inducer of the EpRE-mediated gene transcription, was partially inhibited by staurosporine and is thus partially mediated by PKC (Fig. 5.2A). Other pathways are apparently also contributing to this transcriptional activation, explaining why PKC inhibitors do not inhibit tBHQ induced luciferase expression completely. Our findings suggest that PKC is not involved in the EpRE-mediated luciferase induction by flavonoids. This is concluded from the fact that 10 nM staurosporine, which is sufficient to inhibit 60% of tBHQ-mediated luciferase activation, did not affect EpRE-mediated luciferase induction by quercetin or kaempferol.

Another proposed mechanism for Nrf2 release from Keap1 suggests a direct oxidative modification of Keap1 by inducers of EpRE-mediated gene transcription. Keap1 contains several reactive cysteine residues and disulfide bridge formation between two neighbouring Keap1 monomers holding Nrf2 in complex is proposed to result in the release of Nrf2 (Dinkova-Kostova *et al.* 2005b). In this way, Keap1 might act as a direct sensor for oxidative stress. Consistently, EpRE-mediated gene expression is reported to be a responsive to oxidative type inducers such as ROS and electrophiles. Since flavonoids tend to act as antioxidants rather than oxidants, at first glance an oxidative stress mechanism seems not relevant to explain the induction of Nrf2 release from Keap1 by flavonoids. However, flavonoids have been described to display pro-oxidant activity, after donating electrons either by antioxidant action, enzymatic oxidation or autooxidation (Awad *et al.* 2001; Galati *et al.* 2001; Boersma *et al.* 2002). Flavonoids, especially the ones with a catechol or phenol moiety have the potential to oxidize to quinones or semiquinones resulting in redox cycling and ROS production as well as in thiol, DNA and protein alkylation (Awad *et al.* 2001; Walle *et al.* 2003; Galati and O'Brien 2004; van der Woude *et al.* 2005a). Although the pro-oxidant action of flavonoids is generally considered as unfavourable, the results of the present study indicate that the pro-oxidant action of flavonoids is actually of importance for their inducing activity of an EpRE-mediated response, an effect that can be considered beneficial. This could be concluded from the fact that the E_{HOMO} of the 21 tested flavonoids correlates with their induction factor of the EpRE-mediated gene transcription (Fig. 5.3). E_{HOMO} models the ease of a molecule to donate an electron and has been shown to correlate with the reduction potential which characterizes the ease of oxidation of a compound. Our result is in line with a study reporting the E_{HOMO} of 34 inducers, belonging to 9 different classes, but not including flavonoids, to correlate with the induction of NQO1 enzyme activity in Hepa-1c1c7 cells upon 24 hours exposure to these inducers (Zoete *et al.* 2004).

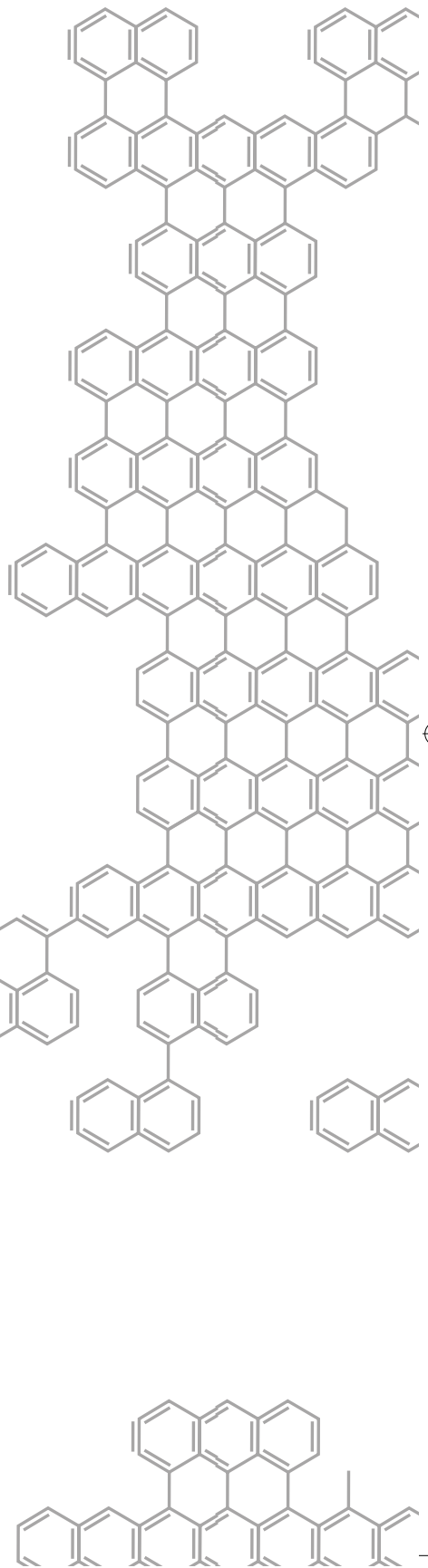
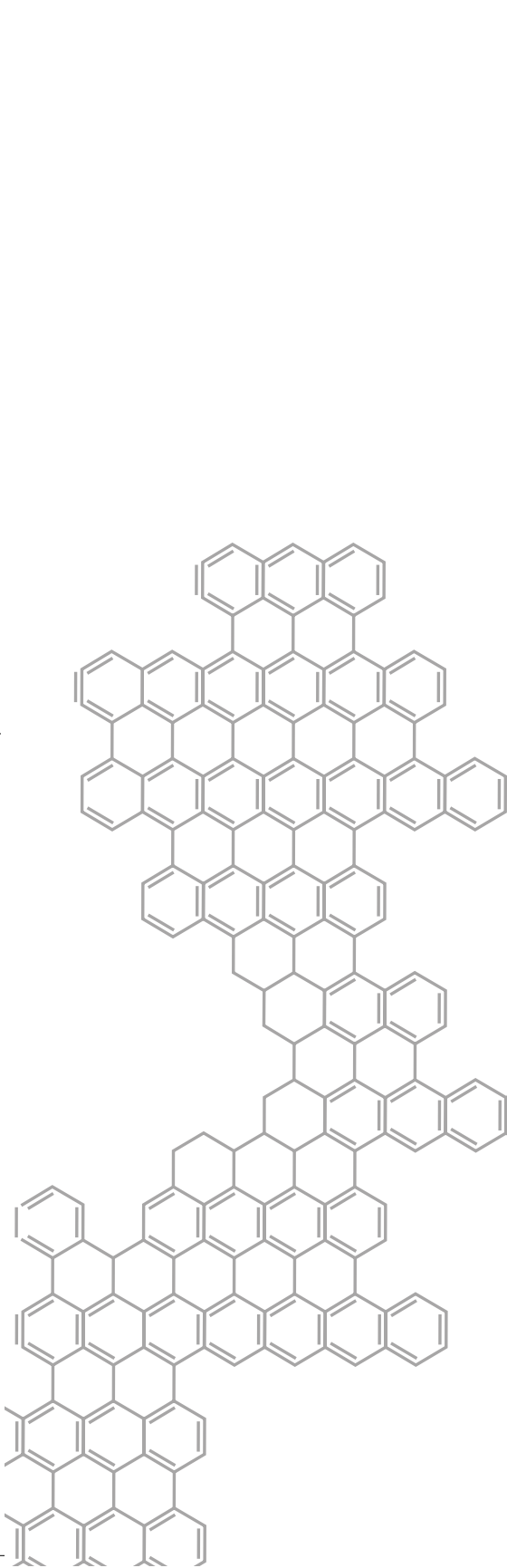
The observation that the inducing activity of flavonoids correlates with their redox properties explains the importance of the presence of a 3-hydroxyl group for their EpRE-mediated response (Table 5.1). Hydroxylation at the 3 position strongly increases the E_{HOMO} , and thus the ease of a flavonoid to donate electrons, while the overall degree of hydroxylation only influences its redox behaviour marginally.

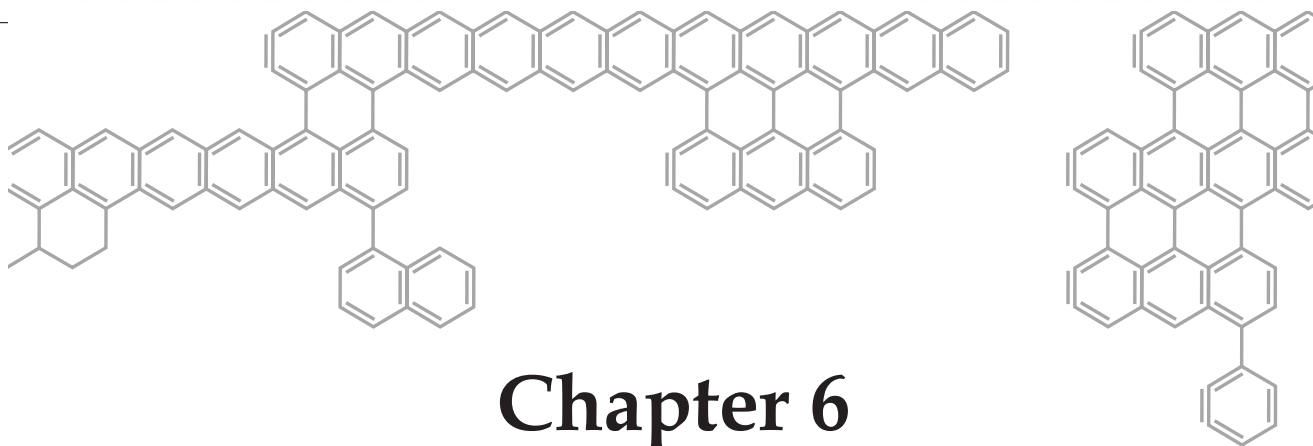
Another line of evidence provided in the present study that supports the conclusion that the pro-oxidant action of flavonoids is essential for their inducing effect of EpRE-mediated response, results from studies characterizing the consequences of modulating the intracellular GSH levels for flavonoid-induced EpRE-mediated gene induction. GSH is essential for maintaining reducing conditions and the reduced state of protein thiols by scavenging reactive free radicals and electrophiles and/or by regeneration of thiol molecules upon their oxidation (Meister and Anderson 1983). Preincubation of the EpRE-LUX cells with NAC to generate increased intracellular GSH levels, resulted in a decrease in the EpRE-mediated gene transcription by quercetin, kaempferol, and fisetin and also by the standard inducer tBHQ (Fig. 5.5). In contrast, reducing the cellular GSH levels by BSO, significantly increased the flavonoid-mediated response (Fig. 5.5). Taken together, these data provide evidence that EpRE-mediated gene transcription by flavonoids is based on their pro-oxidant activity. This finding is in line with other literature reports, suggesting that the pro-oxidant action of flavonoids rather than their antioxidant activity is important for their anticancer properties (Hadi *et al.* 2000; Azam *et al.* 2004).

The precise mechanism by which the pro-oxidant chemistry of flavonoids mediates the EpRE induction is not known but may be related to either ROS production and/or direct alkylation by the flavonoid (semi)quinones resulting in disruption of the Keap1-Nrf2 complex and release of Nrf2 (Dinkova-Kostova *et al.* 2005a; Dinkova-Kostova *et al.* 2005b; Hong *et al.* 2005b). In the latter case, flavonoid quinones might, similar to triterpenoids (Dinkova-Kostova *et al.* 2005b), directly react with cysteine residues of Keap1, leading to conformational changes and Nrf2 release. A direct flavonoid quinone Keap1 interaction could be dependent on steric constraints and thus be dependent on steric parameters of the flavonoid such as its Van der Waals volume. In line with this suggestion, the use of the Van der Waals volume as a second descriptor for the quantitative structure activity relationship for the prediction of the induction factors of EpRE-mediated gene transcription, improves the correlation with the experimental data (Fig. 5.4).

Another possible pathway for the flavonoid-induced activation of EpRE-mediated gene transcription is the Ah receptor (AhR) signaling pathway. Recently it was reported that Nrf2 is regulated by the AhR (Miao *et al.* 2005) and interacting networks between Nrf2 and AhR are increasingly discovered (Kohle and Bock 2006). Although quercetin and kaempferol are reported to be monofunctional inducers (Yannai *et al.* 1998), which mean that they only induce the EpRE-mediated gene expression without the involvement of AhR signaling, other studies report flavonoids to be bifunctional inducers (Fahey and Stephenson 2002; Dinkova-Kostova *et al.* 2004). A structure-based study should be performed to define which flavonoids are inducing EpRE-mediated gene transcription without involvement of the AhR and which flavonoids require AhR signaling, e.g. to (i) generate metabolites or (ii) to generate ROS which in turn induces EpRE-mediated gene transcription.

Altogether, this study demonstrates that the pro-oxidant activity of flavonoids can contribute to their health-promoting activity by inducing important detoxifying enzymes, pointing at a beneficial effect of a supposed toxic chemical reaction.

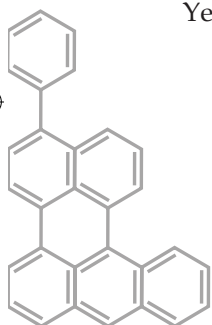




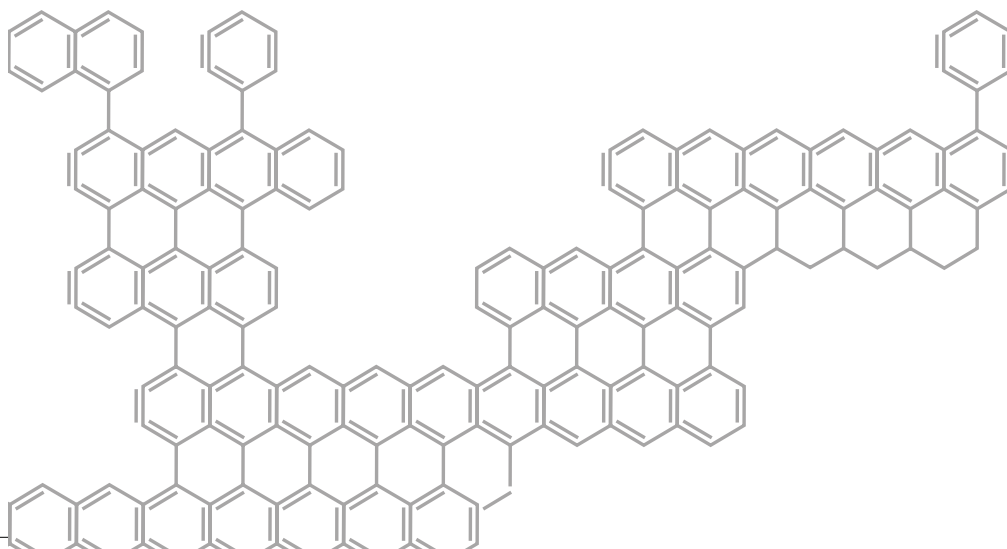
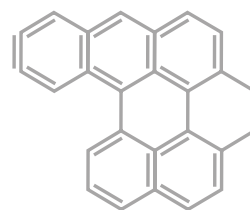
Chapter 6

Quercetin covalently modifies and inactivates Keap1, the key repressor of transcription factor Nrf2

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Submitted to *Antioxidant & Redox Signalling*



Abstract

Transcription of detoxifying enzyme systems, mediated by the electrophile-responsive element (EpRE), plays an important role in reducing the risk of degenerative diseases including cancer. Bioactive dietary compounds, like flavonoids are potential cancer-chemopreventive agents due to their ability to induce detoxifying enzymes via the EpRE transcription enhancer element. EpRE-mediated gene transcription is controlled by the antioxidant transcriptional master regulator Nrf2. Under unstressed conditions, Nrf2 is repressed and destabilised by binding to Keap1. This study shows that quercetin, an important flavonoid in the human diet, can activate Nrf2-controlled EpRE-mediated gene transcription by modifying its repressor Keap1. Radioactive binding studies with purified Keap1 reveal the covalent interaction between quercetin and Keap1. Furthermore, evidence is presented that quercetin can modify Keap1 inside cells, resulting in the dissociation of the Keap1-Nrf2 complex and the formation of Keap1 polymers. Taken together, this study is first to show Keap1 modification by quercetin, resulting in the switch-on of chemopreventive gene transcription activation.

Introduction

Induction of phase II enzymes, which protect cells against the damage of electrophiles and reactive oxygen species, is a major strategy of reducing the risk of diseases like cancer (Kwak *et al.* 2004). Transcription of genes encoding phase 2 enzymes like glutathione S transferases, UDP-glucuronosyltransferases, γ -glutamylcysteine synthetase, NAD(P)H: quinone oxidoreductase 1, heme oxygenase-1, epoxide hydrolase, leukotriene B4 dehydrogenase and aldehyde dehydrogenase, is regulated by the electrophile-responsive element (EpRE) (Chen and Kong 2004). The EpRE is an enhancer element binding certain transcription factors and mediating their transactivation function, of which the nuclear factor-erythroid 2-related factor 2 (Nrf2) is most prominent (Jaiswal 2004). Nrf2 is an unstable protein, which maintains a low basal level of cytoprotective gene expression under homeostatic conditions (Itoh *et al.* 2003). Keap1 (Kelch-like erythroid cell-derived protein with CNC homology-associated protein 1) is an adaptor protein, which regulates the stability of Nrf2 by linking Nrf2 with CuI3-BTB^{Keap1}E3 ligase (Kobayashi *et al.* 2004; Tong *et al.* 2006b) leading to its ubiquitination and degradation. However, a broad range of chemoprotective compounds are capable to induce the escape of Nrf2 from Keap1-mediated degradation, resulting in its translocation to the nucleus and activation of the expression of its target genes. The mechanism by which Keap1-mediated degradation of Nrf2 is inhibited by chemoprotectants is not yet known. The most commonly accepted theory is the direct reaction of oxidative compounds with the Keap1-Nrf2 complex, leading to the release of Nrf2 and activation of EpRE-mediated gene expression (Zhang and Hannink 2003; Wakabayashi *et al.* 2004; Egger *et al.* 2005; Zhang 2006;).

Keap1 is a thiol rich protein (Itoh *et al.* 1999), which comprises 5 domains; the N-terminal region (NTR), the BTB protein-protein interaction motif (BTB), the intervening region (IVR), the double glycine repeat (DGR) and the C-terminal region (CTR). Among the many Keap1 cysteines, Cys257, Cys273, Cys288, Cys297 and Cys613 are the most reactive towards electrophiles (Dinkova-Kostova *et al.* 2002). Cys273 and Cys288, which are located in the IVR of the protein, are essential for Keap1-dependent ubiquitination and Keap1-mediated repression of Nrf2-dependent transcription under basal conditions (Zhang and Hannink 2003; Levonen *et al.* 2004; Wakabayashi *et al.* 2004; Kobayashi *et al.* 2006). Furthermore, Cys151 plays a critical role in electrophile signalling. Modification of Cys151 by an electrophile is required for down-regulation of Nrf2 ubiquitination (Zhang and Hannink 2003; Egger *et al.* 2005; Zhang 2006). In cells exposed to reactive chemicals or oxidative stress, Nrf2 is no longer targeted for ubiquitin-dependent degradation at the DGR of Keap1, resulting in the increase of the steady-state levels of Nrf2, leading to the activation of Nrf2-dependent gene expression (Kobayashi and Yamamoto 2006).

Not all bioactive compounds that induce phase 2 enzymes through EpRE-mediated gene expression are oxidative compounds. Previously, we showed that also flavonoids, commonly known to have electron donating antioxidant properties (Williams *et al.*

2004), can activate EpRE-mediated gene expression (Myhrstad *et al.* 2002; Boerboom *et al.* 2006; Lee-Hilz *et al.* 2006). Flavonoids are important key compounds in fruits and vegetables, suggested to protect against many diseases, like cancer and oxidative stress-related chronic diseases (Hollman and Katan 1997; Chen and Kong 2004; Williams *et al.* 2004; Scalbert *et al.* 2005). Activating EpRE-mediated responses, leading to gene expression of detoxifying enzymes is an important property of flavonoids to be considered as health-promoting. Earlier we showed that the pro-oxidant activity of flavonoids is responsible for the inducing effect of EpRE-mediated gene expression (Lee-Hilz *et al.* 2006). Especially flavonoids with a catechol moiety like quercetin, have the potential to form quinones or semiquinones resulting in redox cycling and ROS production as well as in thiol, DNA and protein alkylation (Hollman and Katan 1997; Awad *et al.* 2001; Walle *et al.* 2003; Galati and O'Brien 2004; van der Woude *et al.* 2005a).

Here we have investigated whether flavonoids can modify Keap1 thiols resulting in the observed activation of Nrf2-regulated gene expression. As a model compound we choose quercetin, an important flavonoid in our diet. Quercetin was recently shown to enhance the EpRE-mediated gene transcription through stabilization of Nrf2 and reducing the level of Keap1 protein (Tanigawa *et al.* 2007). Our studies support these findings and reveal for the first time that the activation of EpRE-mediated gene expression by quercetin is due to Keap1 S-quercetinylation, leading to Keap1 polymerisation and reduction of the posttranscriptional level of the Nrf2 repressor.

Material & Methods

Expression and purification of Keap1

The full-length cDNA copy of mouse Keap1 was inserted between the *Bam*HI and the *Xho*I site of the pET23a(+) expression vector fused to a C-terminal His₆-tag. The correct orientation and sequence of the resulting construct was confirmed by nucleotide sequencing. Keap1-His₆ was expressed in *E. coli* BL21(DE3) cells, grown in LB medium at 28°C for 8 hours. To prevent *in vitro* protein aggregation and precipitation by disulfide bond formation upon cell lysis, 5 mM dithiothreitol (DTT) was added to the lysis buffer (20 mM sodium phosphate pH 8). Cells were disrupted with a precooled French press and the Keap1 recombinant protein was purified on a His-GraviTrap 1 mL pre-packed column (GE Healthcare). After concentration with an Amicon ultra-filtration device, Keap1-His was stored in 20 mM sodium phosphate pH 8 with 5 mM tris[2-carboxyethyl] phosphine (TCEP) to maintain protein solubility.

Preparation of FLAG-Keap1 construct and Keap1-EpRE-LUX cells.

The full length cDNA from mouse Keap1 was inserted between the *EcoRI* and *BamHI* site of a p3xFLAG-CMVTM-10 vector (Sigma). The correct orientation and sequence of the inserted gene was confirmed by nucleotide sequencing. Hepa-1c1c7 cells, a kind gift from Dr. M.S. Denison (University of California, Davis, CA, USA), were cultivated in alpha-Modified Eagle's Medium, supplemented with 10% FCS and 50 µg/mL gentamicin, and cells were maintained in a humidified atmosphere with 5% CO₂ at 37°C. At a confluency of 50-70%, Hepa-1c1c7 cells were transfected with the p3xFLAG-CMVTM-10-Keap1 construct, using a standard calcium phosphate-DNA co-precipitation transfection method (Sambrook *et al.* 1989). The next day the transfected cells were trypsinized, replated and exposed to culture medium containing 0.5 mg/mL antibiotic G418 (Duchefa Biochemie, Haarlem, The Netherlands) allowing the selection of resistant transfectants and the formation of clonal colonies. The G418-resistant clones were picked and propagated in medium containing G418. The clone with the highest Keap1 expression, as revealed by Western Blot analysis, was selected for further studies. These wild type cells will be further addressed to as Keap1-Hepa1-WT cells.

The p3xFLAG-CMVTM-10-Keap1 construct was also co-transfected with a pPUR selection vector (Clontech) into the EpRE(hNQO1)-LUX reporter cell line (Boerboom *et al.* 2006), which are Hepa-1c1c7 cells containing the reporter vector pTI(hNQO1-EpRE)Luc⁺ carrying the EpRE from the human NQO1 gene regulatory region between -470 to -448 (5'-AGT CAC AGT GAC TCA GCA GAA TC-3') coupled to a luciferase reporter gene (designated EpRE-LUX cells).

Clones were selected for resistance to puromycin to select for the presence of p3xFLAG-CMVTM-10-Keap1 construct co-transfected with pPUR, and on the basis of their resistance to G418 clones were also selected for maintenance of the reporter vector pTI(hNQO1-EpRE)Luc⁺. The G418- and puromycin resistant clones were picked and propagated. The clone with the highest Keap1 expression, as revealed by Western Blot analysis, was selected for further studies. These cells will be further addressed to as Keap1-EpRE-LUX cells.

EpRE-LUX assay

EpRE-mediated induction of gene expression in EpRE-LUX cells and the newly transfected Keap1-EpRE-LUX cells by quercetin was tested using the EpRE-LUX luciferase reporter gene assay as described previously (Boerboom *et al.* 2006).

[4-¹⁴C]-Quercetin binding to Keap1

For binding of quercetin to Keap1 *in vitro*, 100 μ L (15 μ g) partially purified Keap1 was incubated with 200 μ M [4-¹⁴C]-quercetin (52.9 mCi/mmoL, Chemsyn Science Laboratories, USA, a kind gift from Dr. J.M.M. van Amelsvoort from Unilever Research Vlaardingen) for 8 hours at room temperature. Samples were taken and used for non-reducing sodium dodecyl sulfate polyacrylamide gelelectrophoresis (SDS-PAGE) analysis.

For binding of quercetin to Keap1 in cells, a confluent bottle (75cm²) of stably transfected Keap1-Heap1-WT cells was trypsinized and cells were diluted in 1 mL of alpha-Modified Eagle's Medium without supplementation. 200 μ M [4-¹⁴C]-quercetin was added to the cell suspension and incubated for 30 min at room temperature. A sample was taken and stored at -80°C for further SDS-PAGE analysis. Before SDS-PAGE analysis, exposure medium of the sample was removed and cells were washed with 50 mM Tris-HCl, containing 150 μ M NaCl (pH 7.4). After cell lysis by three cycles of freezing and thawing using liquid nitrogen and a 37°C waterbath, the supernatant was incubated with 200 μ L ANTI-FLAG M2 agarose affinity gel (Sigma) for 2 hours. Gel beads were collected by centrifugation, washed with 50 mM tris-HCl, containing 150 mM NaCl (pH 7.4) and boiled with SDS-PAGE sample buffer without the addition of β -mercaptoethanol before SDS-PAGE analysis.

Electrophoretic protein separation was performed on non-reducing 12% SDS-PAGE gels. Keap1 was detected with colloidal Coomassie Blue (Invitrogen) staining, Western Blot analysis and radioactive imaging.

Western Blot analysis

After SDS-PAGE, proteins were electrophoretically (8 hours, 20 V) transferred to a polyvinylidene difluoride (PVDF) membrane (Whatman). The membrane was blocked with 5% milk in PBS buffer and probed with anti-FLAG (Sigma) or anti-Keap1 (Santa Cruz) overnight at 4°C. After treatment with the horseradish peroxidase conjugated anti-goat secondary antibody (Santa Cruz) for 45 min at room temperature, immunostained proteins were detected by enhanced chemiluminescence with Western Blotting luminol reagent (GE Healthcare).

LC-MS-MS analysis

For LC-MS-MS analysis, 100 μ L of (15 μ g) purified Keap1-His solution was incubated with 200 μ M quercetin for 8 hours at room temperature. After SDS PAGE and staining with colloidal Coomassie Blue (Invitrogen), the gel region containing the His-tagged Keap1 band was cut out and gel pieces were minced into 1 mm cubes and washed with 50% acetonitrile / 50 mM NH₄HCO₃ (pH 8). 10 μ L of 50 mM DTT was added to reduce the cysteines of Keap1. After 1 h incubation, DTT was replaced by 100 mM

iodoacetamide and the incubation was continued for another hour in the dark. Gel pieces were dried and rehydrated with 20 μ L trypsin (50 ng/ μ L sequencing grade, Roche). Tryptic peptides were collected and analysed with liquid chromatography (LC) separation and mass spectrometry (MS) detection (LCQ classic, Thermo electron, San Jose, CA, USA). Peptides (20 μ L) were concentrated on a 0.10 x 32 mm Prontosil 300-3-C18H (Bischoff, Germany) pre-concentration column and separated on a 0.10 x 200 mm Prontosil 300-3-C18H analytical column with an acetonitrile gradient (10 to 35% acetonitrile in water with 1mL/L formic acid in 50 min) at a flow of 0.5 μ L/min. Downstream of the column, an electrospray potential of 1.8 kV was applied via an Upchurch conductive union (M-572) allowing direct contact with the eluent. Full scan positive mode MS spectra with 3 microscans were measured between m/z 350 and 1400. MS-MS scans of the three most abundant peaks in the MS scan were recorded in data-dependent mode and analyzed with Bioworks 3.2 with the following filter criteria: Δ Cn > 0.08, Xcorr > 2 for charge state 1+, Xcorr > 1.5 for charge state 2+ and Xcorr > 3.3 for charge state 3+ (Peng *et al.* 2003). The *E.coli* K12 database used was downloaded from the European Bioinformatics Institute website (<http://www.ebi.ac.uk/integr8>) in August 2006 to which the following protein sequences were added: BSA (P02769, bovine serum albumin precursor), trypsin (P00760, bovin), trypsin (P00761, porcine), keratin K2C1 (P04264, human) and keratin K1CI (P35527, human).

As for glutathione (GSH) incubation with quercetin, 1mM GSH was incubated with 200 μ M quercetin, 0.1 μ M horseradish peroxidase (HRP) and 200 μ M H₂O₂ for 30 min at 30°C and the sample was subjected to liquid chromatography separation and mass spectrometry detection as described above.

Results

Binding of [4-¹⁴C]-quercetin to purified Keap1

Purification of recombinant Keap1-His₆ from *E.coli* BL21(DE3) by metal-affinity chromatography resulted in a nearly pure monomeric protein preparation as judged by non-reducing SDS-PAGE analysis (Fig. 6.1A).

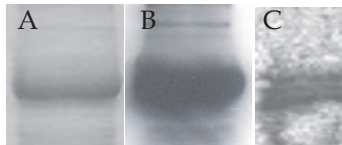


Figure 6.1: Non-reducing SDS-PAGE of purified Keap1 protein (70 kDa) after incubation with [4-¹⁴C]-quercetin. **A** Coomassie Brilliant Blue staining, **B** Western Blot analysis with Keap1 antibody and **C** radioactive imaging.

Western Blot analysis with specific Keap1 antibody confirmed the presence of the Keap1 protein in the single protein band visible at about 70 kDa (Fig. 6.1B). Incubations of [4-¹⁴C]-quercetin with purified Keap1 and subsequent non-reducing SDS-

PAGE analysis clearly showed that Keap1 interacts with this flavonoid (Fig. 6.1C). Performing the SDS-PAGE analysis in the presence of β -mercaptoethanol did not result in the dissociation of quercetin from Keap1 (data not shown). Figure 6.1C also shows that the Keap1 protein band, identified with Western Blot analysis (Fig. 6.1B), consists of several bands in close proximity. This suggests that more than one quercetin molecule can bind to Keap1 *in vitro*, resulting in modified Keap1 protein molecules of different mass.

LC-MS-MS analysis of Keap1

LS-MS-MS analysis was performed to study the binding of quercetin to Keap1 in further detail. First, the exact mass increase of quercetin binding to cysteines was determined by aid of glutathione (GSH). This γ -Glu-Cys-Gly tripeptide is reported to form covalent adducts with quercetin (van der Woude *et al.* 2006). Figure 6.2A shows the chromatogram of GSH after incubation with quercetin in the presence of horseradish peroxidase (HRP) and H_2O_2 .

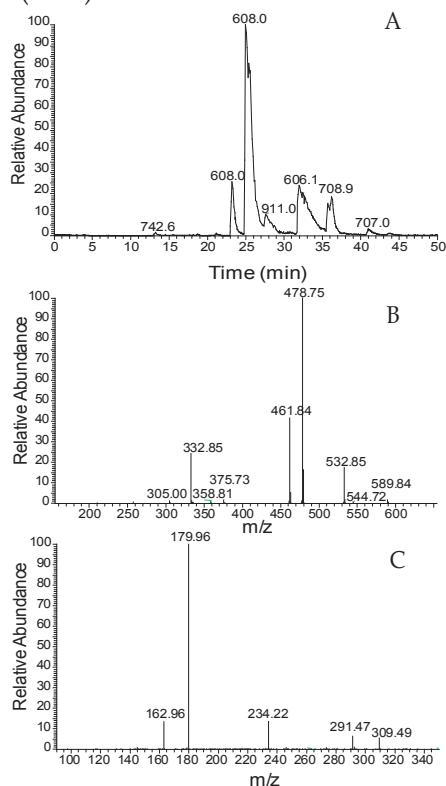


Figure 6.2: LC-MS analysis of the reaction of GSH with quercetin. **A** Covalent adduct formation between quercetin and GSH; **B** Fragmentation pattern of quercetin-modified GSH; **C** Fragmentation pattern of free GSH.

The mass observed for the main peak eluting after 26 min ($m/z = 608$) is in perfect agreement with the expected increase in mass when GSH reacts stoichiometrically with quercetin quinone methide formed from quercetin upon its oxidation by HRP/ H_2O_2 (Boersma *et al.* 2000; van der Woude *et al.* 2006). From the fragmentation patterns of GSH, with (Fig. 6.2B) and without (Fig. 6.2C) quercetin/HRP/ H_2O_2 incubation, a mass increase of 299 after quercetin binding to GSH can be confirmed. Thus, quercetin modification of Keap1 protein, the mass spectra of quercetin-treated Keap1 cysteine containing peptides had to be searched for a mass increase of 299, representing the mass of quercetin bound to a cysteine of Keap1.

Purified Keap1, incubated in the absence and presence of quercetin, was subjected to SDS-PAGE, treated with trypsin and analysed with LC-MS-MS (Fig. 6.3A).



Figure 6.3: Peptides of Keap1 protein measured with LC-MS-MS, derived from the purified Keap1 incubated without **A** and with **B** quercetin/HRP/ H_2O_2 . Detected peptides are coloured in grey with Cys151, Cys273 and Cys288 highlighted in black. Peptides, which were not detected anymore after quercetin incubation, are highlighted in grey.

The protein coverage of the peptide analysis was on average 40%. The peptides containing Cys151, Cys273 and Cys288, reported to be involved in binding of Keap1 inducers, were clearly detectable in the non-treated protein. In the quercetin-treated protein, we could not detect Keap1 peptides with a mass increase of 299. However, after incubation of Keap1 with quercetin, the peptides containing Cys151 and Cys273 were not detectable anymore (Fig. 6.3B). Furthermore, 3 other peptides and another cysteine containing peptide (Cys226) were also not detectable after quercetin incubation (Fig. 6.3B).

Expression of Keap1 in Hepa-1c1c7 cells

Hepa-1c1c7 cells were stably transfected with Keap1 inserted in a p3xFLAG-CMVTM-10 vector. Keap1 expression of the transfected cells was detected by Western Blot analysis with Keap1 and Flag antibody. Western Blot analysis of the cell extracts with Flag antibody clearly showed the insert present in the transfected cells but not in the wild-type cells (Fig. 6.4A).

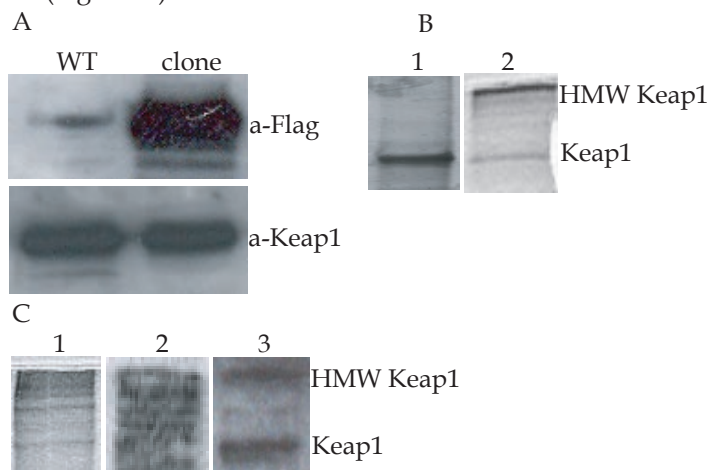


Figure 6.4: **A** Western Blot analysis of wild type cells (WT) and stably Keap1 transfected Hepa-1c1c7 cells (clone) with Flag and Keap1 antibody. **B** SDS-PAGE separation of the cell lysate of Keap1 transfected cells without (1) and with (2) [$4\text{-}^{14}\text{C}$]-quercetin incubation of the cells, immunoprecipitated with ANTI-FLAG M2 agarose beads. **C** SDS-PAGE separation of the cell lysate of Keap1 transfected cells after [$4\text{-}^{14}\text{C}$]-quercetin incubation of the cells with Coomassie Brilliant Blue staining (1), radioactive imaging (2) and Western Blot analysis with Keap1 antibody (3).

However, Western Blot analysis with Keap1 antibody showed that, although a strong promoter was used to drive the expression of the introduced Keap1 cDNA sequence, no overexpression of Keap1 in the transfected cells was observed (Fig. 6.4A). This indicates a cellular process of either down-regulation or degradation of Keap1, hindering its overexpression.

When cell lysates were incubated with ANTI-FLAG M2 agarose beads, Keap1 was bound to the beads and could be detected with Coomassie Blue staining (Fig. 6.4B(1)). This result was confirmed with Western Blot and LC-MS-MS analysis (data not shown). Although minor unspecific binding of other proteins to the ANTI-FLAG M2 agarose beads occurred, this immunoaffinity procedure is suitable for a fast one step Keap1 purification from cell cultures.

Binding of [4-¹⁴C]-quercetin to Keap1 in cells

Radioactive labelled [4-¹⁴C]-quercetin was incubated with Hepa-1c1c7 cells, stably transfected with Keap1 in a p3xFLAG-CMVTM-10 vector. Figure 6.4C shows the intracellular binding of [4-¹⁴C]-quercetin to Keap1. Keap1 cells were incubated with [4-¹⁴C]-quercetin for 30 min, with (1) presenting the non-reducing SDS-PAGE gel with Coomassie Blue staining of the total cell extract. This analysis shows the presence of various proteins ranging from high molecular mass down to 60 kDa. Fig. 6.4C(2) presents the radioactive image of the SDS-PAGE gel. The radioactive image of the cell lysate after quercetin binding shows quercetin binding to various proteins bands with high intensity. Radioactivity could be detected mainly at high molecular mass, but also at a molecular mass of 70 kDa where Keap1 would migrate. The Western Blot analysis, shown in Fig. 6.4C(3), shows the presence of Keap1 at 70 kDa and at high molecular mass. The amount of both forms of Keap1 is approximately equal, based on the intensities of the bands, detected by Western Blot analysis. These results suggest that quercetin can bind to Keap1 inside cells and that this binding leads to the formation of Keap1 polymers, with molecular mass greater than 150 kDa.

Furthermore, Fig. 6.4B shows the SDS-PAGE results of Hepa-1c1c7 cells, transfected with Keap1 in a p3xFLAG-CMVTM-10 vector, incubated without (1) and with (2) [4-¹⁴C]-quercetin and immunoprecipitated with the ANTI-FLAG M2 agarose beads after cell lysis. The SDS-PAGE analysis with Coomassie Blue staining shows the presence of Keap1 with a molecular mass of 70 kDa. Proteins with high molecular mass after quercetin treatment are present, which are not present without quercetin incubation (Fig. 6.4B). The Western Blot detection with Keap1 antibody proves that the high molecular weight band is containing Keap1 protein (data not shown). The radioactive image of the SDS-PAGE gel shows only some intensity in the high molecular mass range but not around 70 kDa, because apparently nearly all Keap1 protein was modified by quercetin to HMW Keap1 (Fig. 6.4B(2)), within the two hours incubation of the cell lysate with the ANTI-FLAG M2 agarose beads.

Activation of EpRE-controlled gene expression by quercetin in EpRE-LUX cells transfected with Keap1

The FLAG-Keap1 construct was stably transfected into EpRE-LUX reporter cells already containing an EpRE-controlled luciferase reporter gene stably integrated in the genomic DNA. The quercetin-induced EpRE-mediated gene expression in the original EpRE-LUX cells was compared with that of 2 clones of our newly transfected Keap1-EpRE-LUX cells. Fig. 6.5 shows the induction of EpRE-mediated induction of gene expression in the different cells lines, induced by quercetin.

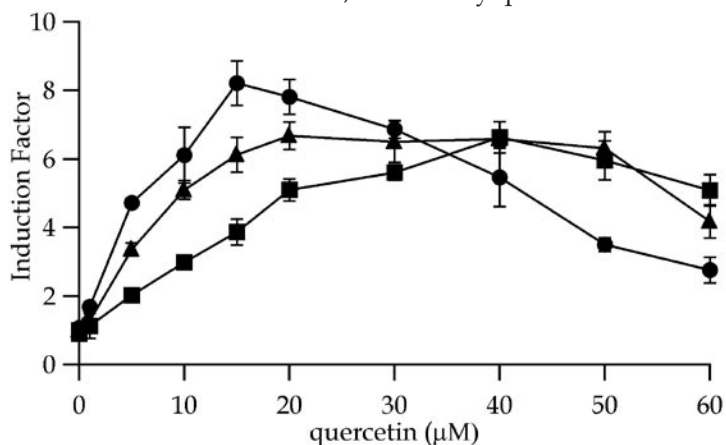


Figure 6.5: Induction of EpRE-mediated gene transcription by quercetin in the original EpRE-LUX cells (●), and in Keap1-EpRE-LUX cells (clone 1 -■- and clone 2 -▲-). Data are presented as means with standard error based on six independent measurements.

Quercetin shows a concentration-dependent luciferase induction in the concentration range tested (0-60 μM) in the EpRE-LUX cells and in the 2 clones of stably transfected Keap1-EpRE-LUX cells (Fig. 6.5). In EpRE-LUX cells a maximal EpRE-mediated response of 8-fold was observed, while both cells lines, transfected with Keap1 show a maximal induction factor of 7-fold. No significant difference could be observed between the Keap1-transfected and non-transfected EpRE-LUX cells.

Discussion

Flavonoids are health-promoting bioactive compounds, present in various fruits and vegetables. We recently showed that flavonoids induce the EpRE-mediated expression of phase II biotransformation enzymes, resulting in the protection of cells against xenobiotics (Boerboom *et al.* 2006; Lee-Hilz *et al.* 2006). Several studies have shown that Keap1 serves as a repressor for the activation of Nrf2 regulated-EpRE-mediated gene expression (Kobayashi *et al.* 2004). The EpRE-mediated gene expression by quercetin was recently shown to function via up-regulation of Nrf2 and repression of Keap1 (Tanigawa *et al.* 2007). However, the precise mechanism of Keap1 repression and Nrf2 induction is not known. Electrophilic modification of Keap1 thiols is proposed to result in the dissociation of Nrf2 from the Keap1-Nrf2 complex, thus enabling Nrf2 nuclear translocation and EpRE activation (Kobayashi *et al.* 2006; Zhang 2006). Our previous study showed that the pro-oxidant action of flavonoids is the reason for their ability to induce EpRE-mediated gene expression (Lee-Hilz *et al.* 2006). Here we investigated whether quercetin, a model flavonoid compound, can modify Keap1 thiols *in vitro* and in intact cells.

Our study shows that quercetin can bind to Keap1 (Fig. 6.1), triggering the earlier reported phase II gene induction (Lee-Hilz *et al.* 2006). Quercetin can oxidize to quinones in cells (van der Woude *et al.* 2005a), resulting in S-quercetynylation of Keap1 thiols. Radioactively labelled [4-¹⁴C]-quercetin can bind to purified Keap1 (Fig. 6.1) and this binding also occurs inside cells (Fig. 6.4C). Beside Keap1, quercetin can also react with other proteins inside cells, without any observed specificity (Fig. 6.4C). This is in accordance with studies from Kaldas and coworkers (Kaldas *et al.* 2005), who showed quercetin binding to proteins like human serum albumin, apo-transferrin and cytochrome C. The ability of quercetin to interact with proteins might be important for some of its numerous biological activities and underlines the complexity of quercetin-mediated signal transduction and protein modification in living cells.

Intracellular quercetin binding to Keap1 results in the formation of high molecular mass Keap1 (Fig. 6.4B). This observation is in line with recent results (Tanigawa *et al.* 2007) and with previous studies where it was shown that Keap1 polymerises upon incubation with tert-butylhydroquinone and N-iodoacetyl-N-biotinylhexylenediamine (Zhang and Hannink 2003; Hong *et al.* 2005b). The conversion of Keap1 to high molecular forms is triggered by specific thiol adduct formation and is linked to the polyubiquitination of Keap1 (Hong *et al.* 2005b). Quercetin-induced polymerization of Keap1 is not observed with purified protein (Fig. 6.1) and no high molecular mass Keap1 formation is observed without quercetin incubation (Fig. 6.4B). This shows that Keap1 polymerization is not a spontaneous process but occurs through a distinct quercetin-induced mechanism inside cells. Our results support a quercetin-induced oxidative mechanism of covalent modification of Keap1, similar to quercetin binding to GSH. Nevertheless, immunoprecipitated Keap1 showed weak quercetin binding. This indicates that quercetin triggers the covalent modification of Keap1, but that

this binding is reversible in time, as also shown for GSH-querctetin adducts (Awad *et al.* 2003).

Although it is assumed that the modification of Keap1 by inducers triggers a switching of ubiquitination from Nrf2 to Keap1, thus stabilizing Nrf2 and allowing de novo synthesized Nrf2 to translocate to the nucleus (Eggler *et al.* 2005; Kobayashi and Yamamoto 2006), recent studies show substantial differences between inducers. Quercetin reduces the Keap1 level through the formation of modified Keap1 rather than through 26S proteasome-dependent degradation mechanisms (Tanigawa *et al.* 2007). Proteasomal turnover of Nrf2 is inhibited by quercetin and Nrf2 is upregulated through transcriptional and posttranscriptional mechanisms (Tanigawa *et al.* 2007). The mechanism of the Keap1 downregulation by quercetin needs to be further investigated.

Identification of the target amino acid residues that are involved in the quercetin-Keap1 interaction was not straightforward. Although a good coverage of tryptic peptides of the purified Keap1 protein was obtained by LC-MS, revealing the most important cysteines, no modifications with quercetin could be assigned. LC-MS-MS analysis of the reaction between quercetin and GSH showed that it is possible to measure quercetin cysteine-adducts (Fig. 6.2). However, for the S-quercetinylation of GSH, the quercetin quinone formation was triggered by a reaction with HRP/H₂O₂, which is reported to occur *in vivo* (van der Woude *et al.* 2005a). For the quercetin modification of Keap1, HRP was not added to form quercetin quinones, because HRP oxidized the cysteines of Keap1 (data not shown). Therefore, 8 hours incubation of quercetin with purified Keap1 was chosen to allow autooxidation of quercetin to quercetin quinones (Makris and Rossiter 2000) and the subsequent S-quercetinylation of Keap1. However, the protein-quercetin conjugates are labile and reversible in time (Awad *et al.* 2003; van der Woude *et al.* 2005a). This complicated the *in vitro* quercetin-Keap1 binding and product formation for LC-MS-MS detection.

Indirect evidence for quercetin modification of Keap1 was obtained from comparison of the LC-MS results for native Keap1 and the modified protein. After incubation of Keap1 with quercetin the peptides harbouring Cys151 and Cys273 were not detectable anymore (Fig. 6.3). Furthermore, three other peptides, including the one with Cys226 were also missing (Fig. 6.3). Although it has been stated that Cys151 is not important for the functioning of mouse Keap1 (Wakabayashi *et al.* 2004), this suggests that quercetin might react with Cys151 and Cys273, and that Cys288 is less sensitive for quercetin modification. This would be in agreement with the notion that Keap1 adduct formation is highly dependent on the chemistry of the modifier and not simply a function of thiol reactivity (Hong *et al.* 2005a).

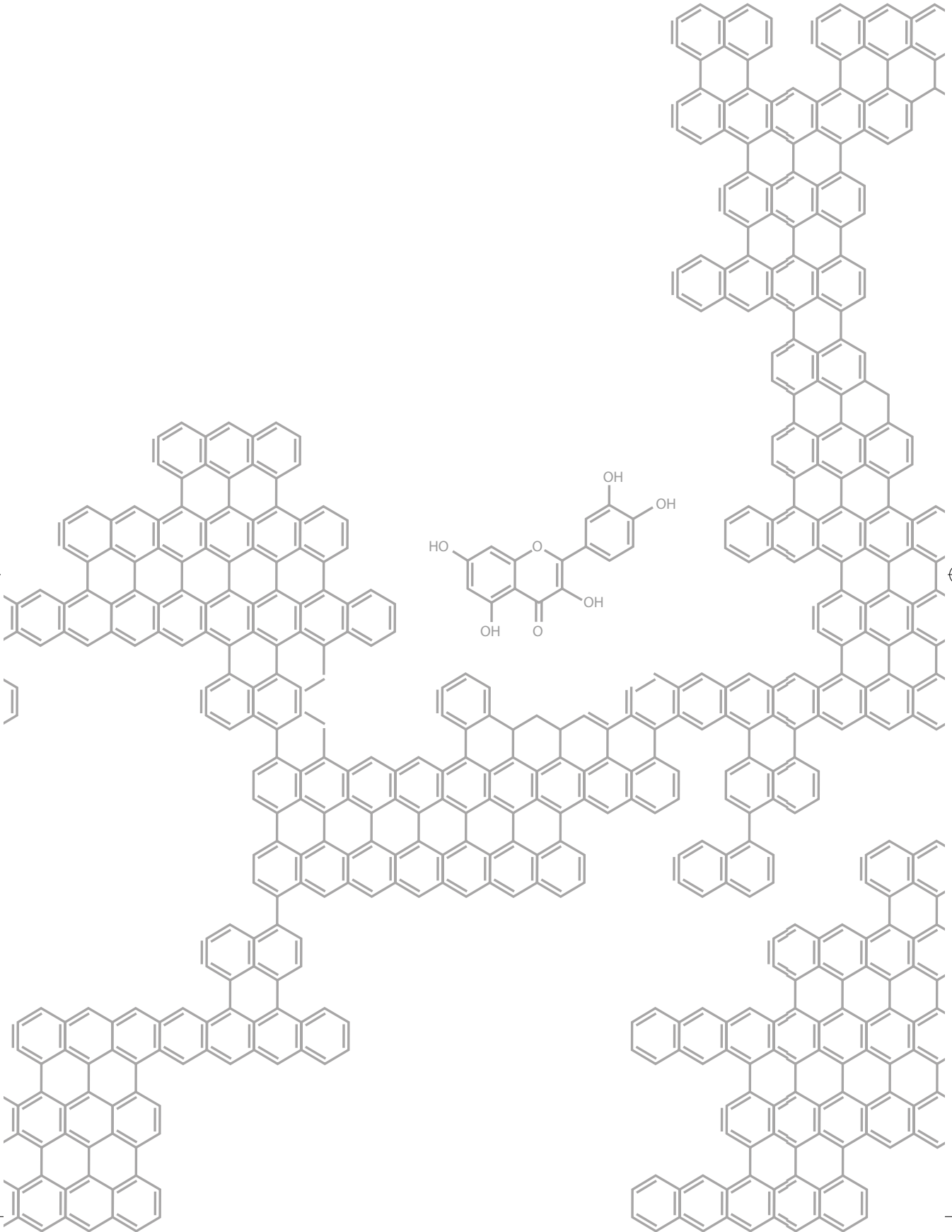
Finally, the results of the present study reveal an intracellular regulation of Keap1. No stable cell line with over-expressed Keap1 could be obtained (Fig. 6.4A), resulting in quercetin-induced EpRE-mediated gene transcription in the Keap1-transfected

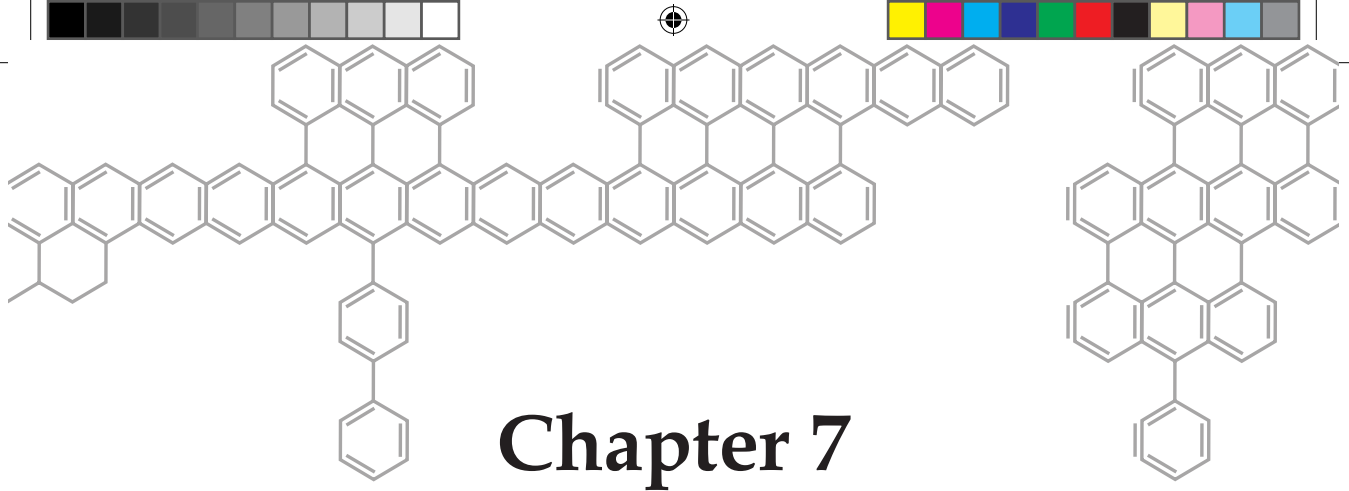


Keap1 S-quercetininylation

EpRE-LUX cells lines that was comparable to that in the non-Keap1-transfected EpRE-LUX cells (Fig. 6.5), despite the use of the strong CMV promotor to drive the expression of the transfected Keap1 allele. Earlier reports showed that *in vitro* overexpression of Keap1 leads to increased ubiquitination of Nrf2, hindering the EpRE-mediated gene transcription (Kobayashi *et al.* 2002), whereas germ line deletion of the *keap1* gene results in mouse pups' death before weaning (Okawa *et al.* 2006). As Keap1 knock-down results in increased EpRE-mediated luciferase induction (Cullinan *et al.* 2004), overexpression of Keap1 is expected to result in a decrease of the quercetin-induced luciferase response. Our results indicate an intracellular control mechanism, hindering the over-expression of Keap1 in cells.

In summary, this study demonstrates that quercetin can bind to Keap1 and modify Keap1, resulting in Keap1 polymerisation to HMW Keap1 and its inactivation followed by the activation of EpRE-mediated gene expression.

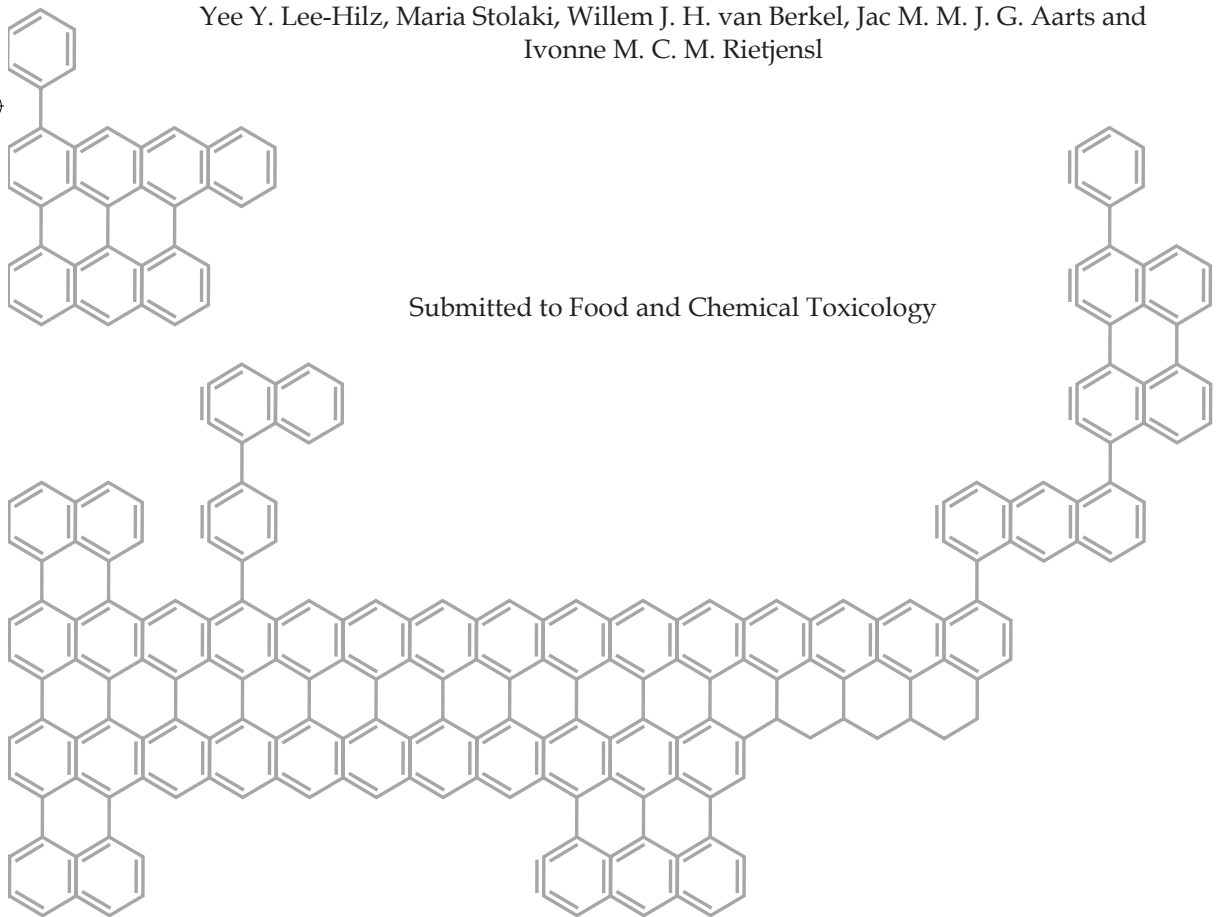




Activation of EpRE-mediated gene transcription by quercetin glucuronides depends on their deconjugation

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Abstract

Quercetin is a flavonoid reported to have health-promoting properties. Due to its extensive metabolism to glucuronides *in vivo*, questions were raised if studies conducted with quercetin aglycone, stating its health-promoting activity, are of actual relevance. Here we show that glucuronides of quercetin, and its methylated forms isorhamnetin and tamarixetin, can induce EpRE-mediated gene expression up to 5-fold. Furthermore, evidence is presented that EpRE-mediated gene induction by these glucuronides involves their deglucuronidation. This indicates, that although quercetin-derived glucuronides are the major metabolites present in the systemic circulation, deglucuronidated derivatives are the active compounds responsible for its beneficial EpRE-mediated gene expression effects.

Introduction

Flavonoids are reported to exert protective effects against many diseases, in particular cancer and oxidative stress-related chronic diseases (Hollman and Katan 1997; Chen and Kong 2004; Walle 2004; Scalbert *et al.* 2005). The cancer-preventive activity of flavonoids has been attributed to, amongst others, the induction of important phase II enzymes (Lee-Hilz *et al.* 2006), which play a central role in the defense system of cells, by detoxifying reactive genotoxic substances and by contributing significantly to the cellular protection against redox cycling and oxidative stress (Chen and Kong 2004; Itoh *et al.* 2004). The transcription of genes encoding detoxifying enzymes such as glutathione S-transferases and NAD(P)H:quinone oxidoreductase 1 (NQO1) (Dinkova-Kostova *et al.* 2005a) was shown to be regulated by the electrophile-responsive element (EpRE), an upstream enhancer element of certain phase 2 genes (Jaiswal 2004).

Quercetin, a flavonoid widely present in fruits and vegetables, is reported to be a strong inducer of EpRE-mediated gene transcription (Boerboom *et al.* 2006; Lee-Hilz *et al.* 2006; Valerio *et al.* 2001). The estimated daily intake of flavonoids ranges up to 1 g/day (Scalbert and Williamson 2000), to which quercetin may contribute up to 40-100% (Hertog 1995). In foods, flavonoids are often present as β -glycosides of the aglycones. Flavonoid metabolism in the body is demonstrated to occur to a significant extent (Day *et al.* 2000; Rechner *et al.* 2002; Arts *et al.* 2004). Upon ingestion, flavonoid glycosides are deglycosylated and the aglycones are metabolized into glucuronide-, sulfate- and methyl-conjugates (Spencer *et al.* 2004). Only a small part of the metabolites is transported to the blood stream and circulates, with flavonoid glucuronides being the major metabolites present in the systemic circulation (de Boer *et al.* 2005; Kanazawa *et al.* 2006).

Since flavonoid metabolites were found in the circulation and these circulating glucuronides, sulfates and methylated forms are believed to be the ones exerting the biological effects (Spencer *et al.* 2004) it is necessary to extend the performed studies, reporting health-promoting activity by flavonoid aglycones with studies on flavonoid phase 2 metabolites. Therefore, the aim of this study is to get insight in the effect of flavonoid metabolism on its capacity to modify a supposed beneficial endpoint. As a model compound we used quercetin and the beneficial endpoint studied was EpRE-mediated gene transcription. The EpRE-mediated gene expression induced by two well-characterized mixtures of quercetin phase 2 glucuronides was characterized in Hepa-1c1c7 cells, stably transfected with a luciferase reporter gene under transcriptional control of the EpRE derived from the human NQO1 gene. The results obtained indicate that the activation of EpRE-mediated gene transcription by the quercetin phase 2 metabolites depends on their cellular deglucuronidation.



Materials & Methods

Materials

Quercetin and ascorbic acid were purchased from Sigma (Steinheim, Germany). 3'-O-methylquercetin (isorhamnetin) and 4'-O-methylquercetin (tamarixetin) were purchased from Extrasynthèse (Genay Cedex, France). Fetal Calf Serum (FCS), Hanks' Balanced Salt Solution (HBSS), phosphate-buffered saline (PBS), trypsin, alpha-Modified Eagle's Medium, gentamicin and G418 were purchased from Gibco Invitrogen Corporation (Breda, The Netherlands) and HPLC-grade acetonitrile was obtained from Lab-Scan Ltd. (Dublin, Ireland).

Cell lines

The human HT29 colon carcinoma cell line was purchased from the American Type Culture Collection (Manassas U.S.A.) and the rat H4IIE hepatocellular carcinoma cell line was purchased from the European Collection of Cell Cultures (ECCC). The Hepa-1c1c7 mouse hepatoma cell line was a kind gift from Dr. M.S. Denison, (University of California, Davis). All cell lines were cultured in alpha-Modified Eagle's Medium, supplemented with 10% FCS and 50 µg/mL gentamicin, and cells were maintained in a humidified atmosphere with 5% CO₂ at 37°C. Hepa-1c1c7 cells were stably transfected with the reporter vector pTI(hNQO1-EpRE)Luc⁺ carrying the EpRE from the human NQO1 gene regulatory region between -470 to -448 (5'-AGT CAC AGT GAC TCA GCA GAA TC-3') coupled to a luciferase reporter gene, as described previously (Boerboom *et al.* 2006). The culture medium of the transfected Hepa-1c1c7 cells was the same as for the wild-type Hepa1-c1c7 cells, containing in addition 0.5 mg/mL G418. These transfected Hepa-1c1c7 cells (original name EpRE(hNQO1)-LUX), containing the luciferase gene under expression regulation of the EpRE from the human NQO1 gene will further be referred to as EpRE-LUX cells.

Quercetin metabolite formation

H4IIE and HT29 cells were exposed to alpha-Modified Eagle's Medium containing 50 µM and 100 µM quercetin respectively. Ascorbic acid (final concentration 1 mM) was added to prevent quercetin auto-oxidation. After 24 hours of exposure samples of the medium were taken. All samples were stored at -80°C until HPLC analysis and further experimentation.

HPLC analysis of quercetin metabolites

The identification of quercetin metabolites was performed as described previously (van der Woude *et al.* 2004). Briefly, HPLC analysis was performed on a Waters M600 liquid chromatography system, using an Alltima C18 5U column (4.6 mm × 150 mm; Alltech, Breda, The Netherlands). Detection was performed between 220 and 445 nm

using a Waters 996 photodiode array detector. Chromatograms used are based on detection at 370 nm. The limit of detection of this HPLC method for quantification of quercetin and its metabolites was 0.1 μ M (injection volume 10 μ L).

EpRE-LUX assay

EpRE-mediated induction of gene expression by quercetin, isorhamnetin and tamarixetin was tested using the EpRE-LUX luciferase reporter gene assay as described previously (Boerboom *et al.* 2006). For the incubations with quercetin glucuronide mixtures the EpRE-LUX luciferase reporter gene assay was slightly modified as follows: monolayers of EpRE-LUX cells in 96-wells view-plates (Corning, 100 μ L/well) were treated with 100 μ L medium containing quercetin glucuronide mixtures formed upon 24 hours incubation of HT29 or H4IIE cells with quercetin. After 24 hours of exposure, cells were washed and analyzed according to the EpRE-LUX luciferase reporter gene assay as described previously (Boerboom *et al.* 2006).

Metabolism of quercetin and quercetin metabolites mixtures by EpRE-LUX cells

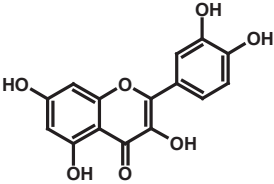
EpRE-LUX cells were exposed to the quercetin metabolite mixtures, formed by HT29 and H4IIE cells (as described above) or to quercetin aglycone for 24 hours. After 24 hours of exposure, samples of the medium were taken and the EpRE-LUX cells were washed with PBS. After addition of 1 mL methanol (65%), cells were harvested and all samples were stored at 80°C until HPLC analysis, as described above.

Results

Metabolism of quercetin

Table 7.1 shows the composition and percentage of quercetin phase 2 metabolites found in the culture medium of HT29 and H4IIE cells after 24 hours incubation in the presence of 100 μ M and 50 μ M quercetin, respectively.

Table 7.1: Quercetin metabolite, presented as % of total peak intensity, formed after 24 hours incubation of quercetin with HT29 or H4IIE cells

| Quercetin | | | |
|---|---|----------|-----------|
|  | | | |
| Compound | | HT29 (%) | H4IIE (%) |
| 1 | 7-O-glucuronosyl-quercetin | 3.7 | 66.9 |
| 2 | 3-O-glucuronosyl-quercetin | 27.1 | 11.7 |
| 3 | 3-O-glucuronosyl 3'-O-methyl-quercetin | 3.3 | n.d. |
| 4 | 3-O-glucuronosyl 4'-O-methyl-quercetin | n.d. | n.d. |
| 5 | 7-O-glucuronosyl 3'-O-methyl-quercetin | n.d. | 9.1 |
| 6 | 7-O-glucuronosyl 4'-O-methyl-quercetin | n.d. | 2.0 |
| 7 | 4'-O-glucuronosyl-quercetin | 39.9 | n.d. |
| 8 | 3'-O-glucuronosyl-quercetin | 7.1 | 10.3 |
| 9 | 4'-O-glucuronosyl 3'-O-methyl-quercetin | 14.9 | n.d. |
| 10 | 3'-O-glucuronosyl 4'-O-methyl-quercetin | 3.3 | n.d. |
| 11 | Quercetin | 0.7 | n.d. |

n.d. = not detected

The major metabolites formed by HT29 cells are 4'-O-glucuronosyl quercetin (40%) and 3-O-glucuronosyl quercetin (27%), while the major metabolite formed by H4IIE cells is 7-O-glucuronosyl quercetin (67%). Upon 24 hours incubation, the residual level of quercetin present in the incubation media was less than 1%.

Activation of EpRE-controlled gene expression by quercetin and quercetin metabolites

Fig. 7.1 shows the induction of EpRE-mediated luciferase expression by quercetin and its methylated metabolites, isorhamnetin and tamarixetin, which are concentration-dependent in the concentration range tested (0-60 μM).

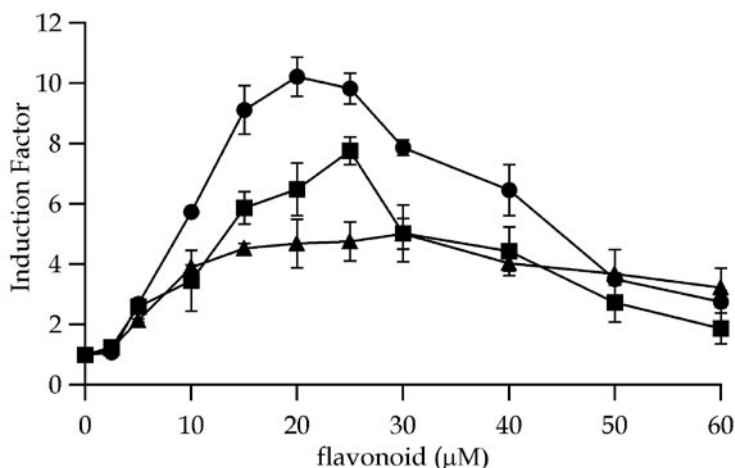


Figure 7.1: Induction of EpRE-mediated gene transcription by quercetin (-●-), isorhamnetin (-■-) and tamarixetin (-▲-). Data are presented as means with standard error based on six independent measurements in one experiment.

Isorhamnetin and tamarixetin show a lower maximal EpRE-mediated response of 8- and 5-fold, respectively as compared to quercetin with a 10-fold maximal induction. Furthermore, the EpRE-mediated induction of gene expression by quercetin phase 2 metabolite mixtures, formed by HT29 and H4IIE cells, as described above, were tested (Fig. 7.2).

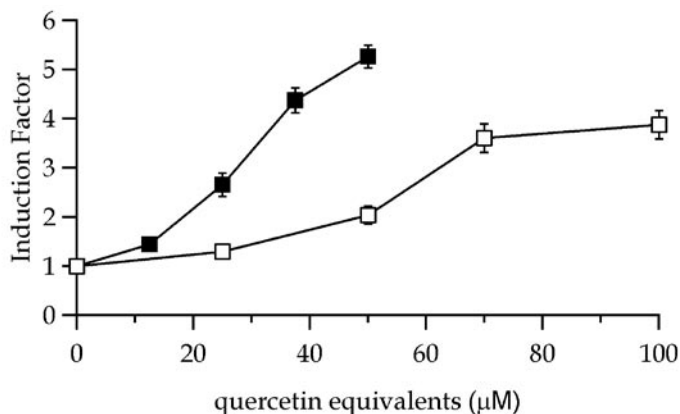


Figure 7.2: Induction of EpRE-mediated gene transcription by quercetin metabolite mixtures formed by HT29 (□) and H4IIE (■) cells. Data are presented as means with standard error based on six independent measurements in one experiment.

Quercetin metabolites show a concentration-dependent increase in luciferase induction, with a maximum induction factor reached of 3.9 for the metabolite mixture formed by HT29 cells and 5.3 for the metabolite mixture formed by H4IIE cells.

Modification of quercetin metabolites by EpRE-LUX cells

To define the fate of quercetin metabolites upon their incubation with EpRE-LUX cells, the level of quercetin metabolites in the EpRE-LUX cell culture medium and the intracellular levels of quercetin and/or its metabolites upon exposure of the EpRE-LUX cells to medium from quercetin-exposed HT29 cells were characterized by HPLC. Fig. 7.3 presents the HPLC chromatograms reflecting the changes in the quercetin metabolite patterns formed by HT29 cells after incubation with EpRE-LUX cells.

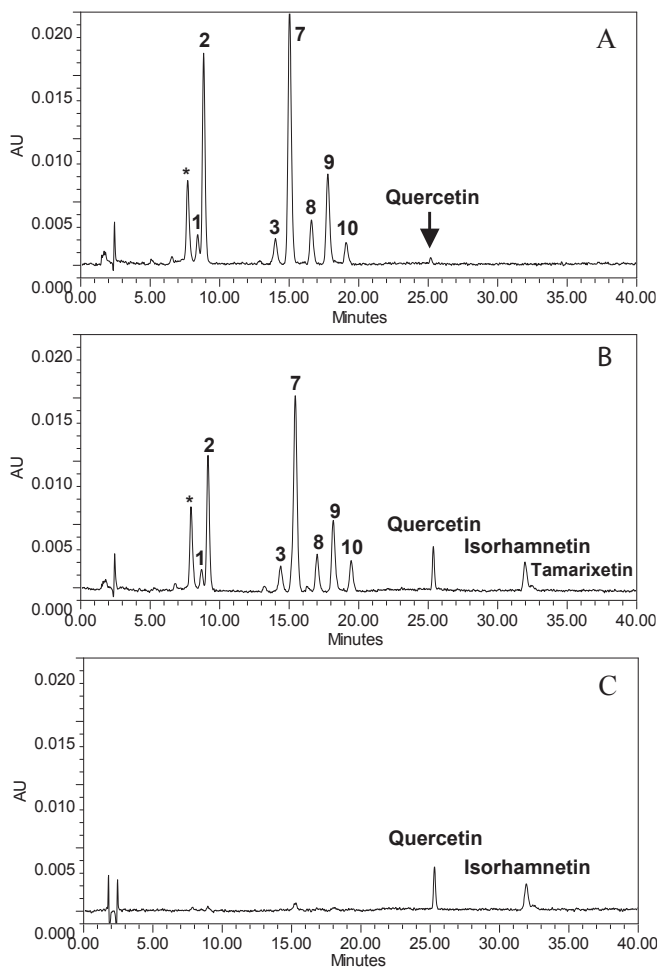


Figure 7.3: HPLC chromatograms demonstrating the fate of the quercetin metabolite mixtures formed by HT29 cells when added to EpRE-LUX cells and representing **A** quercetin metabolite mixture formed by the HT29 cells and added to the EpRE-LUX cells at $t=0$, **B** quercetin metabolite mixture resulting after 24 hours incubation with the EpRE-LUX cells, and **C** intracellular content of EpRE-LUX cells after 24 hours of incubation with the quercetin metabolite mixture. The peak marked with * represents phenol red present in the medium.

Fig. 7.3A presents the HPLC chromatogram demonstrating the metabolite pattern of quercetin after 24 hours incubation with HT29 cells, as already quantified in Table 7.1. Fig. 7.3B shows the chromatogram revealing the quercetin metabolite pattern observed in the medium after 24 hours incubation with EpRE-LUX cells. All quercetin metabolites formed by HT29 cells (Fig. 7.3A) are still present after 24 hours incubation with EpRE-LUX cells (Fig. 7.3B). Furthermore, 3 new peaks are visible with retention times



of 25.4 min, 32.0 min and 32.4 min, which were identified as quercetin, isorhamnetin and tamarixetin respectively (Fig. 7.3B). Fig. 7.3C shows the HPLC chromatogram of the intracellular content of the EpRE-LUX cells after exposure to the quercetin metabolite mixture. Inside the cells, no quercetin glucuronides are detectable, upon the detection limit of the present method, but quercetin and isorhamnetin are clearly present in the intracellular content of the cells.

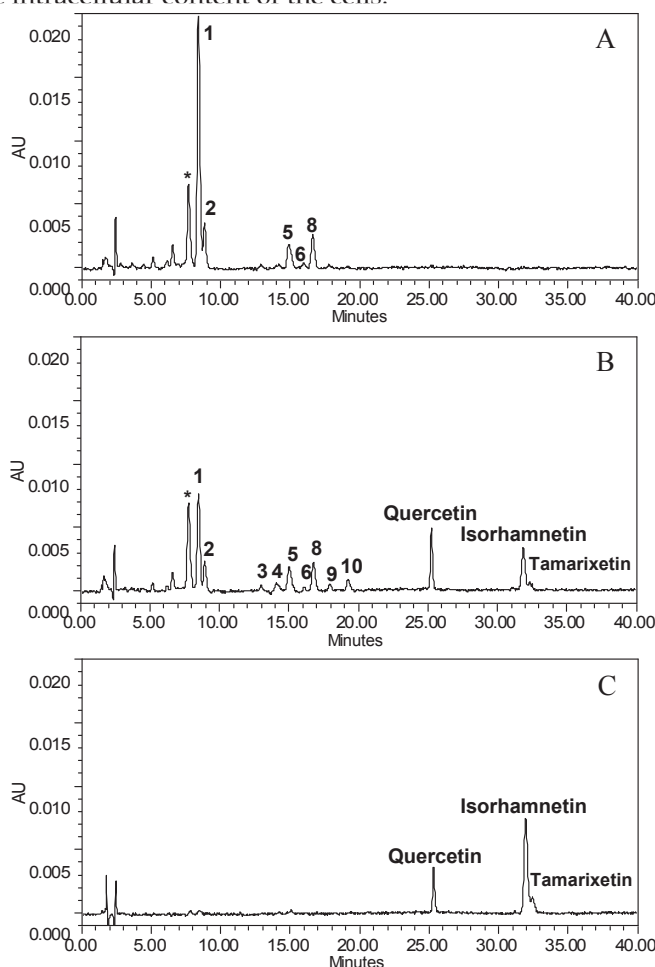


Figure 7.4: HPLC chromatograms demonstrating the fate of the quercetin metabolite mixtures formed by H4IIE cells when added to EpRE-LUX cells and representing **A** quercetin metabolite mixture formed by the H4IIE cells and added to the EpRE-LUX cells at $t=0$, **B** quercetin metabolite mixture resulting after 24 hours incubation with the EpRE-LUX cells, and **C** intracellular content of EpRE-LUX cells after 24 hours of incubation with the quercetin metabolite mixture. The peak marked with * represents phenol red present in the medium.

Fig. 7.4 shows the HPLC chromatograms revealing the changes in metabolite pattern after 24 hours exposure of the EpRE-LUX cells to medium containing the quercetin mixture formed by H4IIE cells, with Fig. 7.4A presenting the quercetin metabolites present in this mixture, as already quantified in Table 7.1. Fig. 7.4B shows the HPLC chromatogram of the quercetin metabolite mixture after 24 hours incubation with EpRE-LUX cells. The main metabolite, 7-O-glucuronidated quercetin decreased significantly accompanied by the formation of several new metabolites, which were identified to be 3-O-glucuronosyl-3'-O-methylquercetin, 3-O-glucuronosyl-4'-O-methylquercetin, 4'-O-glucuronosyl-3'-O-methylquercetin and 3'-O-glucuronosyl-4'-O-methylquercetin. In addition to these glucuronidated metabolites, quercetin, isorhamnetin and tamarixetin are formed (Fig. 7.4B). HPLC analysis of the intracellular content of EpRE-LUX cells reveals the absence of any quercetin glucuronide metabolites but the presence of quercetin, isorhamnetin and tamarixetin within the cells (Fig. 7.4C).

Discussion

The biological action of quercetin is extensively studied and various health claims have been made. Most studies done, claiming a health-promoting effect of quercetin, are performed with quercetin aglycone, while the effect of quercetin phase 2 metabolism has not been taken into account explicitly (Kroon *et al.* 2004). However, flavonoids are metabolized and occur predominantly in plasma and tissues as methyl, sulfate and/or glucuronyl conjugates (Spencer *et al.* 2004). This raises the question, whether conjugated quercetin can perform the same protective action as the unconjugated parent compound. This study shows that quercetin phase 2 metabolites can also activate EpRE-mediated gene expression thereby inducing detoxifying enzymes such as NQO1. Mixtures of glucuronidated quercetin metabolites, formed by HT29 and H4IIE cells, were shown to be able to induce EpRE-mediated gene expression (Fig. 7.2). The maximal induction by the quercetin glucuronide mixtures was 4- to 5-fold which is 40-50%, 50-63% and 80-100% of the maximal level of induction by quercetin aglycone, isorhamnetin and tamarixetin, respectively. HPLC analysis of the metabolite mixtures added to the EpRE-LUX cells clearly shows the absence of free quercetin, isorhamnetin and tamarixetin in the glucuronide mixtures (Fig. 7.3A, 7.4A and Table 7.1). Therefore the induction of EpRE-mediated gene expression by quercetin metabolite mixtures cannot be due to residual quercetin, isorhamnetin or tamarixetin present in these quercetin metabolite mixtures produced by the HT29 and H4IIE cells. However, although the EpRE-LUX cells were only exposed to glucuronidated quercetin metabolites, HPLC analysis of the extracellular medium and the intracellular content of EpRE-LUX cells after 24 hours exposure to the quercetin glucuronide mixtures revealed the presence of non-glucuronidated quercetin aglycone, isorhamnetin and tamarixetin (Fig. 7.3, 7.4). Additional experiments revealed that the intracellular level of quercetin aglycone and its methylated metabolites isorhamnetin and tamarixetin in the EpRE-LUX cells exposed to the quercetin glucuronide mixtures were in the same range as the levels of these compounds in cells exposed to quercetin aglycon (data not shown). Therefore, we conclude that the EpRE-mediated

gene induction by quercetin glucuronide mixtures can be ascribed to the activity of the deglucuronidated analogues and that the EpRE-mediated gene induction by the quercetin glucuronide mixtures is the result of efficient deconjugation of these conjugates at the cellular level. Whether this deglucuronidation occurs inside or outside the cell remains to be established.

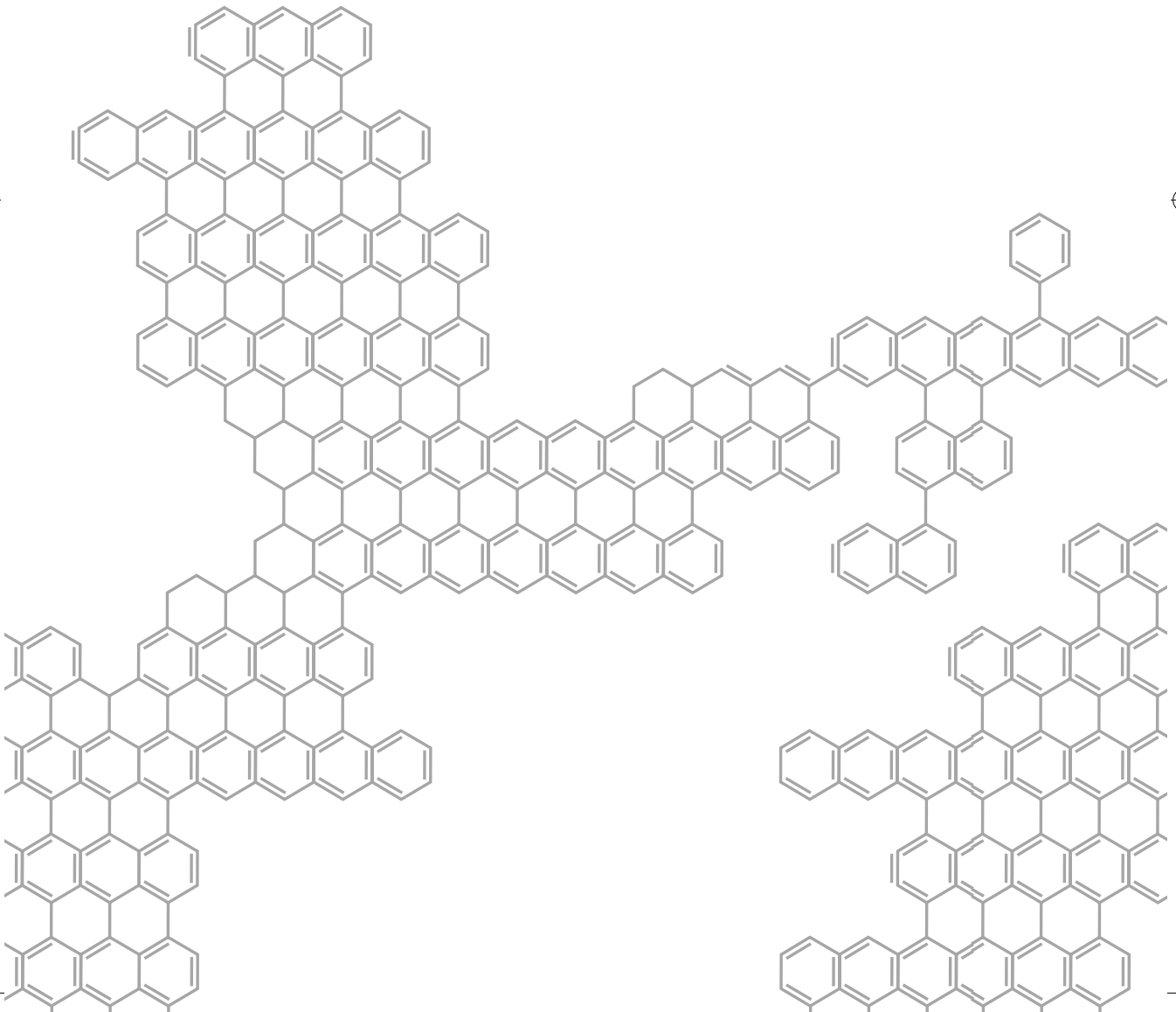
Earlier it has been reported, that quercetin-3-O-glucuronide can be taken up by mouse fibroblast 3T3 cells (Shirai *et al.* 2002). Therefore it is possible that deglucuronidation occurs at the intracellular level. Supporting the intracellular deglucuronidation, O'Leary (O'Leary *et al.* 2003) reported, that quercetin 3-O- β -glucuronide and quercetin 7-O- β -glucuronide were deconjugated in hepatocytes and further metabolized to quercetin 3'-O-sulfate, indicating that quercetin glucuronides can enter cells and that enzymes with β -glucuronidase activity are able to convert quercetin glucuronides intracellularly to the quercetin aglycone. Furthermore, it is assumed that endogenous glucuronidase levels in tissues may enable aglycone formation at the target site in vivo (de Boer *et al.* 2005). It has also been reported that deconjugation of glucuronide conjugates occurs at inflammation sites (Shimoi *et al.* 2000; Shimoi and Nakayama 2005). Taken together, our results, showing the presence of quercetin aglycone and its methylated metabolites tamarixetin and isothamnetin in the extracellular medium and in the intracellular content of EpRE-LUX cells, support the presence of β -glucuronidase(s), responsible for the deconjugation of glucuronidated quercetin derivatives to their deglucuronidated analogues.

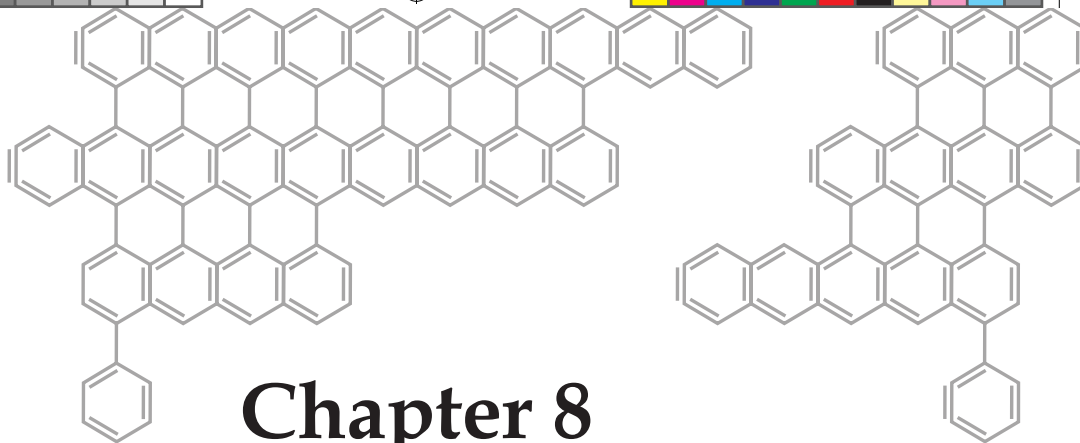
In conclusion, the results of the present study reveal that glucuronidated quercetin metabolites can induce similar health-promoting EpRE-mediated gene expression as shown for quercetin aglycone. Furthermore, this study indicates that although quercetin glucuronides may be the major metabolites present in the systemic circulation, actual biological effects including the EpRE mediated gene induction studied in the present paper, may still be ascribed to the non-glucuronidated derivatives formed at the cellular level.



Bioactivity of quercetin glucuronides

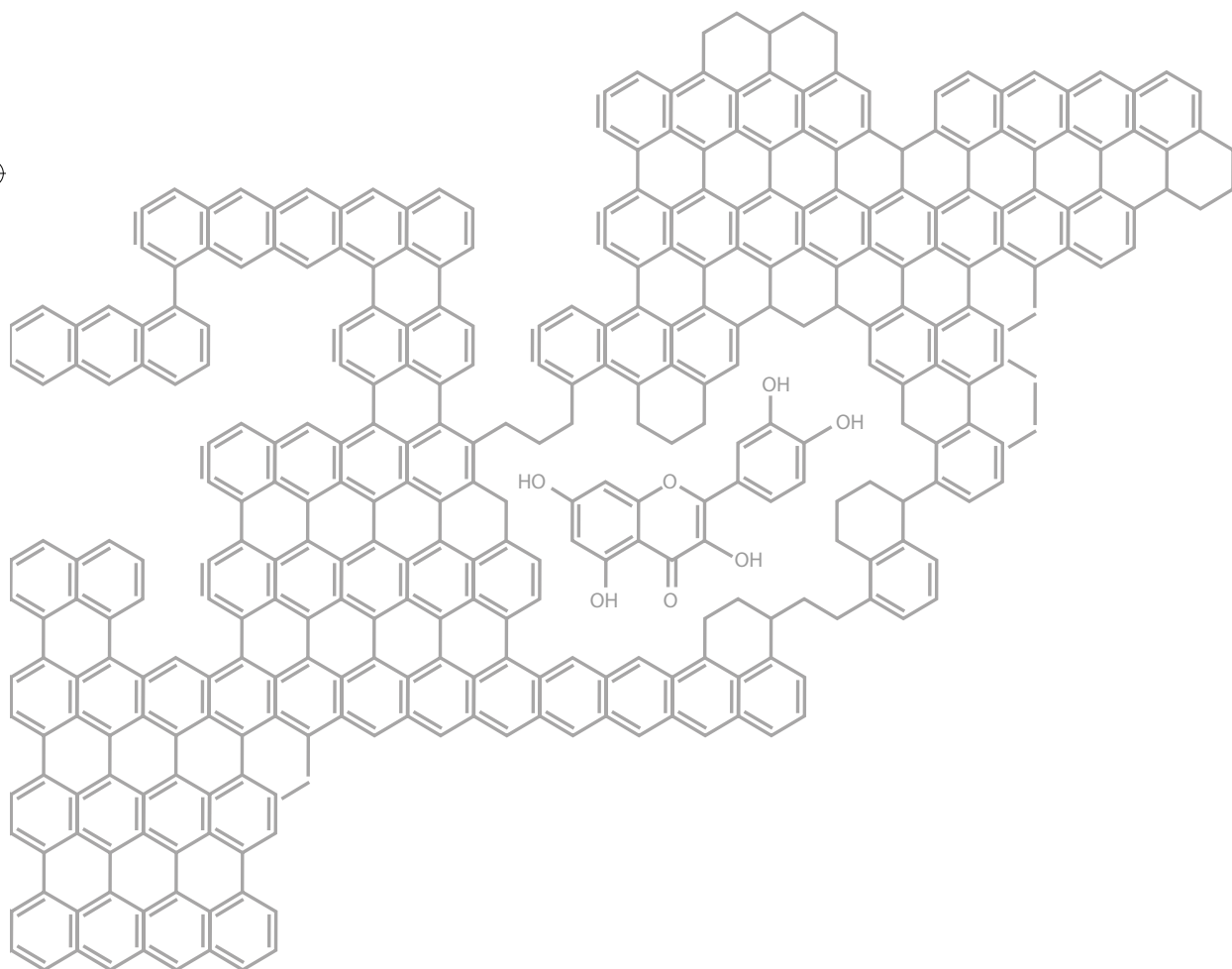
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Chapter 8

Summary and concluding remarks



Flavonoids and NAD(P)H: quinone oxidoreductase 1

Fruit- and vegetable-rich diets have been associated with reduced incidences in cardiovascular disease, neurodegenerative diseases, stroke and certain forms of cancer (Johnsen 2004; Vainio and Weiderpass 2006). Flavonoids are important key compounds in these food items, considered to be health-protecting. They have been reported to protect against diseases through their antioxidant, anti-inflammatory, anti-allergic and antiviral activities (Scalbert *et al.* 2005). Some of the flavonoids were recently applied as functional food ingredients, or put on the market as herbal medicines/nutraceuticals and/or dietary supplements. As a result, the human consumption of flavonoids is expected to increase in the near future.

The protective effect of such bioactive compounds can be accomplished by modulating the activity of enzyme systems responsible for deactivation of chemical carcinogens, like NAD(P)H: quinone oxidoreductase 1 (NQO1). Induction of NQO1 activity is considered a biomarker of anticarcinogenic chemopreventive action, since NQO1 was found to be involved in protection against toxic agents and reactive forms of oxygen (Ross and Siegel 2004). NQO1 detoxifies quinones and nitro aromatics in a single-step two electron reduction, thereby preventing the one electron reduction of quinones by cytochromes P450 and their redox cycling with molecular oxygen generating superoxide radicals, as well as their electrophilic activity resulting in covalent interaction with cellular macromolecules (Ross and Siegel 2004). Individuals homozygous for the C609T NQO1 variant, a polymorphism of the *NQO1* gene resulting in decreased NQO1 activity, show an increased susceptibility to carcinogenesis and xenobiotic-induced toxicity (Siegel *et al.* 1999).

NQO1 and flavonoids, both supposed to be health-promoting factors by themselves have a complex interplay. Several flavonoids are efficient inhibitors of the NQO1 activity *in vitro* (Chapter 2, 3). On the other hand, certain flavonoids act through the electrophile-responsive-element (EpRE), as inducers of NQO1 gene expression, resulting in increased levels of NQO1 protein (Chapter 4, 5, 6, 7). In this summarizing chapter, the outcome of the studies presented in this thesis will be discussed and some future studies will be suggested.

Inhibition of NQO1 activity

NQO1 is a multi-tasking enzyme, involved in the maintenance of the redox-balance in cells. This detoxification enzyme is inhibited by flavonoids. The inhibition of NQO1 by flavonoids represents a mechanism opposing the reported beneficial effects of these phytochemicals. The mode of flavonoid inhibition was studied in detail in **Chapter 2** and **3**.

In **Chapter 2**, inhibition studies with purified NQO1 and molecular docking were performed to describe a model for flavonoid inhibition of human NQO1. Among various flavonoids tested, 7,8-dihydroxyflavone appeared to be the most potent NQO1 inhibitor. The mechanism of inhibition of NQO1 by flavonoids appeared to be competitive towards NADH. Refined docking, followed by CHARMM optimization revealed that 7,8-dihydroxyflavone can bind at the same site as dicoumarol, with the keto group of 7,8-dihydroxyflavone interacting with His161 and Tyr128'. Furthermore, the 7-OH and 8-OH group of this flavone can form hydrogen bonds with Tyr126', an interaction that may contribute to the high affinity of the enzyme for 7,8-dihydroxyflavone.

Although *in vitro* studies with purified NQO1 protein give detailed insight in the inhibition mechanism, they cannot represent the physiological and biochemical conditions in the intact cell, taking the availability of inhibitors and naturally present cofactors into account. Therefore, procedures for assessing enzyme inhibition in living cells are an important tool to study the relevance of inhibition of enzyme-catalysed reactions detected with purified enzymes for the situation in intact cells and thus in the human body.

In **Chapter 3** we developed a new method to study the effects of flavonoids on the cellular activity of NQO1 in intact cells. The principle of this method is based on the resorufin reductase activity of NQO1 and the strong fluorescence of resorufin. The decrease in resorufin fluorescence in time was used to determine NQO1 activity in intact Chinese hamster ovary (CHO) cells. Applying this method, it was found that for all flavonoid inhibitors tested the IC_{50} in intact cells was at least 3 orders of magnitude higher than the IC_{50} in cell lysates. Among the flavonoids tested, 7,8-dihydroxyflavone was the only compound that inhibited NQO1 activity in living cells. However, a high concentration of 7,8-dihydroxyflavone (133 μ M for 50% inhibition) was needed to inhibit NQO1 activity in intact cells, whereas the apparent inhibition constant K_i *in vitro* was 16 nM. The results in **Chapter 3** demonstrate that *in vitro* studies with purified NQO1, or with extracts from disrupted tissues, are of limited value to obtain insight in the situation in living cells. Several cellular factors are responsible for the ineffectiveness of *in vitro* NQO1 inhibitors in living cells. One of them is the high intracellular NAD(P)H concentration. Flavonoids compete with NAD(P)H (**Chapter 2, 3**) thereby inhibiting the transfer of reducing equivalents from NAD(P)H to FAD. Consequently, the high NADPH concentration inside cells,

which is about 0.7 mM in CHO cells (Nagele 1995), prevents the inhibition of NQO1 activity by flavonoids. Assuming competitive inhibition and an apparent K_i value of 16 nM for 7,8-dihydroxyflavone, it can be calculated that the estimated theoretical IC_{50} value for the inhibitor 7,8-dihydroxyflavone in the presence of 0.7 mM of the substrate NADPH is 5 μ M 7,8-dihydroxyflavone. Still, this estimation does not take the NADH concentration inside the cell into account, which can also modulate the intracellular NQO1 activity.

Chapter 2 and 3 showed that although flavonoids possess the potential to inhibit NQO1 activity *in vitro*, the situation in living cells is far more complex. In spite of the fact that flavonoids are omnipresent in the human diet, it is questionable whether flavonoids can reach concentrations in human tissue above 100 μ M, which appear to be required in order to achieve effective intracellular inhibition of NQO1. Reported concentrations of flavonoids (free and conjugated) in human serum and plasma usually reach maximal levels of only up to 10 μ M even after intake of a highly supplemented diet or the intake of highly dosed food supplements (Hollman, *et al.* 1996, Olthof, *et al.* 2000, Walle, *et al.* 2001). However, van der Woude *et al.* (van der Woude *et al.* 2003) estimated that the concentration of free quercetin might possibly reach 100 μ M in the intestinal lumen after ingestion of a quercetin supplement (250-500 mg). However, **Chapter 3** shows, that only 7,8-dihydroxyflavone can inhibit NQO1 activity inside cells at an extracellular concentration of 100 μ M, whereas quercetin was unable to inhibit NQO1 at this concentration. Given the fact that the flavonoid-mediated inhibition of NQO1 proceeds by competitive inhibition towards NAD(P)H it can be concluded that the inhibition of NQO1 in intact cells depends on the intracellular NAD(P)H concentration in these cells. Based on these considerations it is concluded that further studies aimed at better understanding the possible risk of NQO1-flavonoid interaction at increased flavonoid intake should especially focus on the intestinal cells and not on other possible target organs, because only intestinal cells are likely to be exposed to extracellular flavonoid concentrations in a range that could cause inhibitory effects on intracellular NQO1 activity. Our studies show, that upon oral intake of flavonoids in all other organs the inhibition of NQO1 by flavonoids is not likely to be of physiological relevance and is therefore not opposing the reported beneficial effects of these phytochemicals.

Activation of NQO1 gene expression

Flavonoids can exert protective effects by selectively inhibiting or stimulating key proteins in cell signalling cascades and modulating the activity of enzymes, including phase 1 and phase 2 biotransformation enzymes. For example, flavonoids were shown (**Chapter 4, 5, 6, 7**) to induce health-protective gene expression mediated by the electrophile-responsive element (EpRE), a regulatory sequence involved in the co-ordinated transcriptional activation of genes associated with phase 2 biotransformation, such as NQO1.

Flavonoids can also act as ligands for the aryl hydrocarbon receptor (AhR), resulting in activation of the xenobiotic-responsive element (XRE), for the transcription activation of phase 1 enzymes. Although the activation of phase 1 enzymes is generally considered as an adverse effect, due to their ability to activate procarcinogens and convert them to reactive intermediates that can damage DNA, lipids and proteins, these enzymes can also positively participate in the detoxification of xenobiotics and decrease DNA adduct formation (Nebert and Dalton 2006). Beside phase 1 enzymes, AhR also regulates the transcription of chemoprotective enzyme genes like NQO1 and glutathione S-transferase P1 (GSTP1), which both have, in addition to an EpRE, a functional XRE in their regulatory region (Jaiswal 1991; Nebert and Duffy 1997; Ma *et al.* 2004).

It has been proposed that EpRE-mediated gene expression of NQO1 is partly regulated by AhR inducers (Miao *et al.* 2004). Moreover, certain flavonoids have been reported to be bifunctional inducers activating both EpRE- as well as XRE-mediated gene expression. It has been suggested that induction of genes controlled by the Ah receptor is needed to generate the ultimate inducer responsible for the activation of EpRE-mediated gene expression by flavonoids (Miao *et al.* 2004). However conflicting results are reported in the literature on this issue (Yannai *et al.* 1998; Fahey and Stephenson 2002), and therefore it is currently unclear whether activation of upstream AhR-controlled gene expression is really necessary in order to achieve EpRE-mediated gene transcription activation.

To clarify whether the flavonoid-induced EpRE-mediated gene expression is a result of an upstream XRE-mediated gene expression, five flavonoids were tested for their ability to induce XRE- and EpRE-mediated gene expression with the use of the same Hepa-1c1c7 cell line stably transfected with a firefly luciferase reporter gene, respectively under expression regulation of an EpRE from the human NQO1 gene, or an XRE-containing sequence from the mouse cytochrome P4501A1 gene. As described in **Chapter 4**, all tested flavonoids were able to activate EpRE- as well as XRE-mediated gene expression. Although classified as bifunctional inducers, due to their ability to induce phase 1 and phase 2 gene expression, all flavonoids were found to induce EpRE- and XRE-mediated gene expression in a different concentration range, which presents an issue not considered by the current definition of a bifunctional

inducer. At physiological relevant concentrations (1-10 μM), the induction of gene expression via the EpRE transcriptional enhancer element is dominant, leading in particular to elevated levels of detoxifying phase 2 enzymes. Furthermore, these results strongly suggest that EpRE-mediated gene expression induced by flavonoids is not a downstream reaction of XRE-mediated gene expression and illustrate that the bifunctional inducer concept should take into account whether both induction functions are actually simultaneously operational in target cells.

In **Chapter 5**, the mechanism of flavonoid-induced EpRE-mediated gene expression of the NQO1 enzyme was further elucidated. EpRE-mediated gene transcription is controlled by the redox-sensitive transcriptional master regulator Nrf2 which, under unstressed conditions, is repressed by complex formation with the cytoplasmic protein Keap1, preventing Nrf2 to reach the nucleus and bind to EpRE transcriptional enhancer elements of genomic DNA. Induction of EpRE-mediated gene transcription involves the release of Nrf2 from a complex with Keap1, either by an oxidative reaction of the inducer with Keap1, or by protein kinase C (PKC)-mediated phosphorylation of Nrf2. To test the involvement of PKC in the EpRE-mediated gene transcription activation by flavonoids, we used staurosporine as a specific inhibitor of PKC, and studied whether the luciferase induction by tBHQ and flavonoids in EpRE-LUX cells would be inhibited by staurosporine. Inhibition of PKC in EpRE-LUX cells inhibited tBHQ-mediated but not flavonoid-mediated luciferase induction which revealed that PKC is not involved in flavonoid-induced EpRE-mediated gene transcription. Furthermore, the ability of twenty-one tested flavonoids to activate an EpRE-mediated response was found to correlate with their redox properties characterized by quantum mechanical calculations. Flavonoids with a higher intrinsic potential to generate oxidative stress and redox cycling are the most potent inducers of EpRE-mediated gene expression. Modulation of the intracellular glutathione (GSH) level showed that the EpRE-activation by flavonoids increased with decreasing cellular GSH levels and vice versa, supporting an oxidative mechanism. Although the pro-oxidant action of flavonoids is generally considered as unfavourable, the results of **Chapter 5** showed that the pro-oxidant activity of flavonoids can contribute to their health-promoting activity by inducing important detoxifying enzymes, pointing at a beneficial effect of a supposed adverse reaction.

Further support for the pro-oxidant activity of flavonoids being responsible for the activation of EpRE-mediated gene expression is presented in **Chapter 6**. We investigated whether flavonoids can modify Keap1 thiols resulting in the observed activation of Nrf2-regulated gene expression. As a model compound we choose quercetin, one of the most important flavonoids in our diet. **Chapter 6** shows that quercetin can activate Nrf2-controlled-EpRE-mediated gene transcription by modifying its repressor Keap1. Radioactive binding studies with purified Keap1 revealed a covalent interaction between quercetin and Keap1. Furthermore, quercetin can also modify Keap1 inside cells, resulting in the formation of Keap1 polymers. Fig. 8.1 shows a proposed model of quercetin-induced Keap1-Nrf2 complex dissociation.

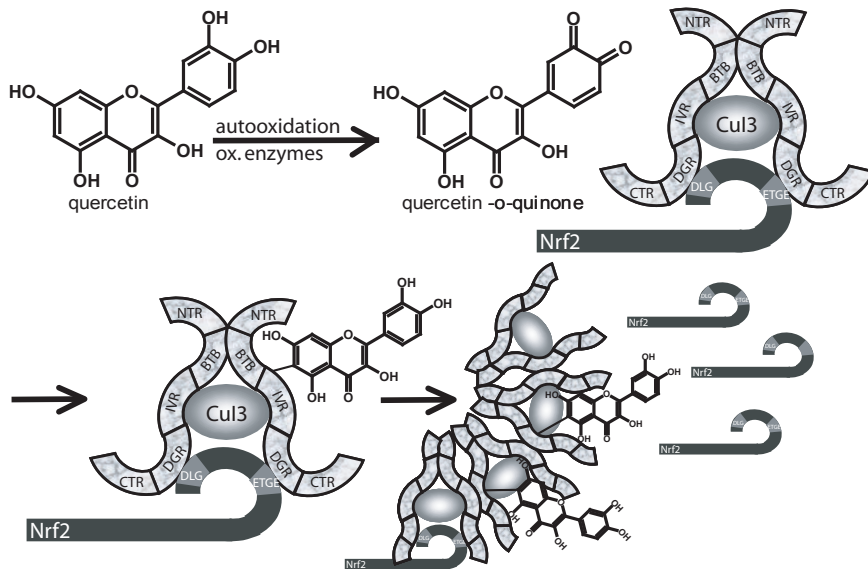


Figure 8.1: Proposed mechanism of quercetin-induced dissociation of the Keap1-Nrf2-complex.

Keap1 is illustrated schematically with its 5 domains (NTR, BTB, IVR, DGR, CTR). While the IVR of Keap1 is responsible for the interaction of the protein with CuI3-based E3 ligase (Kobayashi *et al.* 2004), the DGR motif of the Keap1 protein is essential for Nrf2 binding (Kang *et al.* 2004). Keap1 recruits Nrf2 through binding with the ETGE and DLG motifs in the Neh2 domain of Nrf2 (Tong *et al.* 2006a). Quercetin can oxidize to quinones in cells (van der Woude *et al.* 2005a), resulting in alkylation of Keap1 thiols. The binding of quercetin to Keap1 is assumed to occur at the same binding positions as reported for GSH (Awad *et al.* 2002; van der Woude *et al.* 2005a), of which one possible binding position is depicted in Figure 8.1. This binding of quercetin to Keap1 triggers the aggregation of Keap1. As a result, proteasomal turnover of Nrf2 is inhibited by quercetin and Nrf2-controlled gene transcription is upregulated (Tanigawa *et al.* 2007). However, it is also possible that modification of Keap1 does not result in release of Nrf2 but that the modified Keap1 in complex with Nrf2 is unable to direct newly synthesised cytosolic Nrf2 to degradation through ubiquitination. As a result, *de novo* synthesis of Nrf2 would lead to increased Nrf2 levels and increased Nrf2 mediated-gene transcription.

Taken together, the results in **Chapter 6** revealed that the activation of EpRE-mediated gene expression by quercetin is due to Keap1 S-quercetinylation, leading to Keap1 polymerisation and reduction of the posttranscriptional level of the Nrf2 repressor.

Chapter 4-6 suggest that the health-promoting properties of flavonoids can at least in part be linked to the induction of EpRE-mediated gene expression. These studies were performed with flavonoid aglycones. However, due to the extensive metabolism of flavonoid aglycones to glucuronides *in vivo*, questions were raised if studies conducted with quercetin aglycone, stating its health-promoting activity, are of actual relevance for the *in vivo* situation. Therefore, we investigated, whether conjugated quercetin can perform the same protective action as the unconjugated parent compound. In **Chapter 7** we showed that glucuronides of quercetin, and its methylated forms isorhamnetin and tamarixetin, can induce EpRE-mediated gene expression up to 5-fold as compared to 10 fold by quercetin aglycone. Furthermore, although the EpRE-LUX cells were only exposed to glucuronidated quercetin metabolites, HPLC analysis of the extracellular medium and the intracellular content of EpRE-LUX cells after 24 hours exposure to the quercetin glucuronide mixtures revealed the presence of non-glucuronidated quercetin aglycone, isorhamnetin and tamarixetin. The intracellular level of quercetin aglycone and its methylated metabolites isorhamnetin and tamarixetin in the EpRE-LUX cells exposed to the quercetin glucuronide mixtures were in the same range as the levels of these compounds in cells exposed to quercetin aglycone. Therefore, the EpRE-mediated gene induction by quercetin glucuronide mixtures is ascribed to the activity of the deglucuronidated analogues and the result of efficient deconjugation of the quercetin glucuronides at the cellular level. This indicates that although flavonoid-derived glucuronides are the major metabolites present in the systemic circulation, flavonoids aglycons turn out to be the active compounds responsible for the beneficial EpRE-mediated gene expression effects.

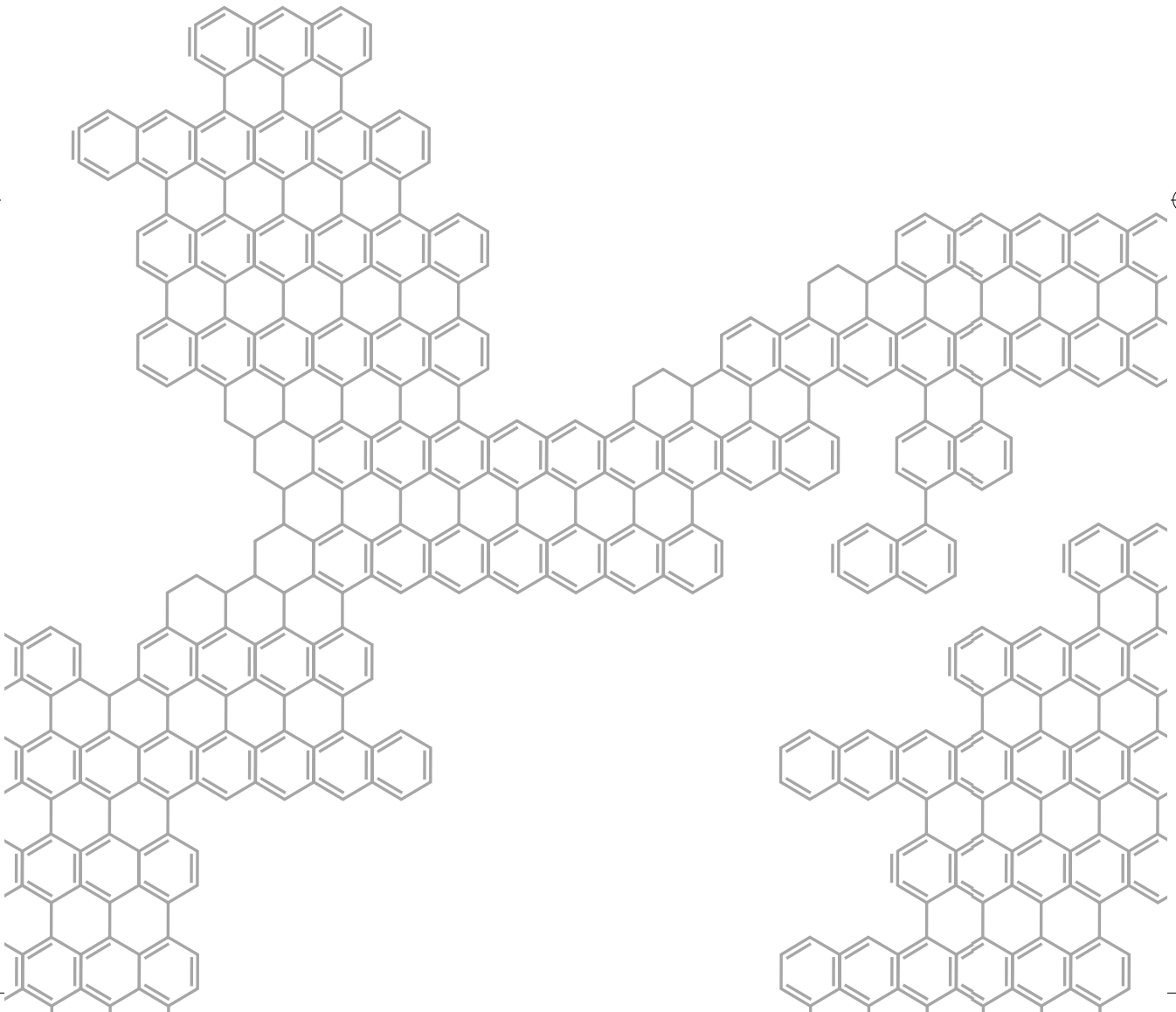
Conclusions

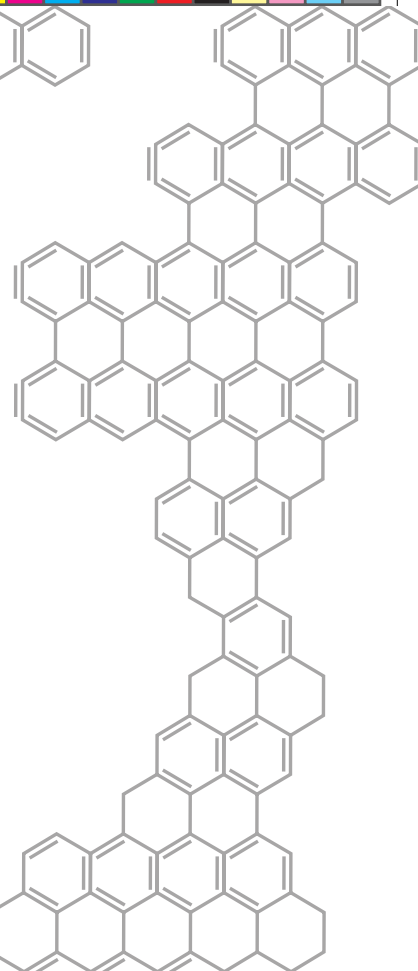
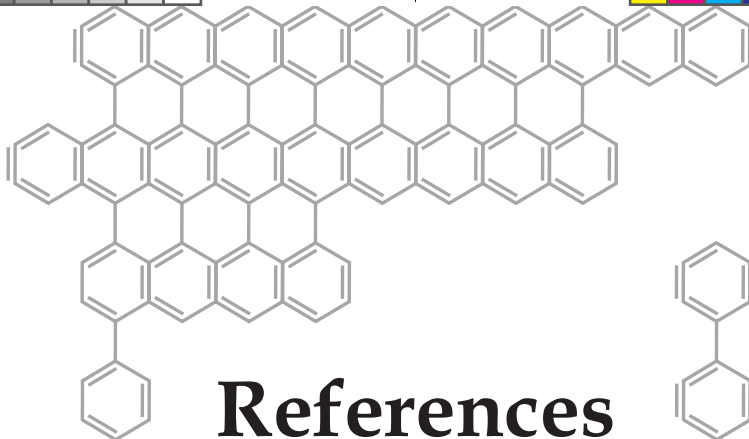
This thesis describes the *in vitro* and *in vivo* interplay between NAD(P)H: quinone oxidoreductase 1 and health-promoting flavonoids. Studies on the protein level showed that flavonoids are able to inhibit NQO1 activity, but this inhibition is not likely to occur in the cellular system due to the competitive nature of the flavonoid inhibitor/NAD(P)H substrate interaction and the high intracellular NAD(P)H concentration. The studies presented in this thesis clearly established that the most important factor in the interplay between NQO1 and flavonoids is not the inhibition of NQO1 by flavonoids but the flavonoid-mediated induction of NQO1 gene expression. Flavonoids and also their glucuronidated metabolites can induce the gene expression of NQO1. Intriguingly, and in contrast to what is generally advertized, the pro-oxidant activity of flavonoids can trigger a beneficial response, in this case the EpRE mediated gene transcription of detoxifying enzymes. The maximal plasma concentrations of flavonoids in humans, reached usually between 1 and 3 h after consumption of flavonoid-rich foods, are between 0.06 and 10 μM for flavonols, flavanols, and flavanones (Manach *et al.* 2005). Flavonoid concentrations of 1 μM can already significantly induce EpRE-mediated gene expression and maximal EpRE-mediated gene expression is reached with flavonoid concentrations up to 20 μM (**Chapter 4, 5**).

Thus, it can be concluded that this chemoprotective effect of flavonoids is most dominant at physiological relevant concentrations.

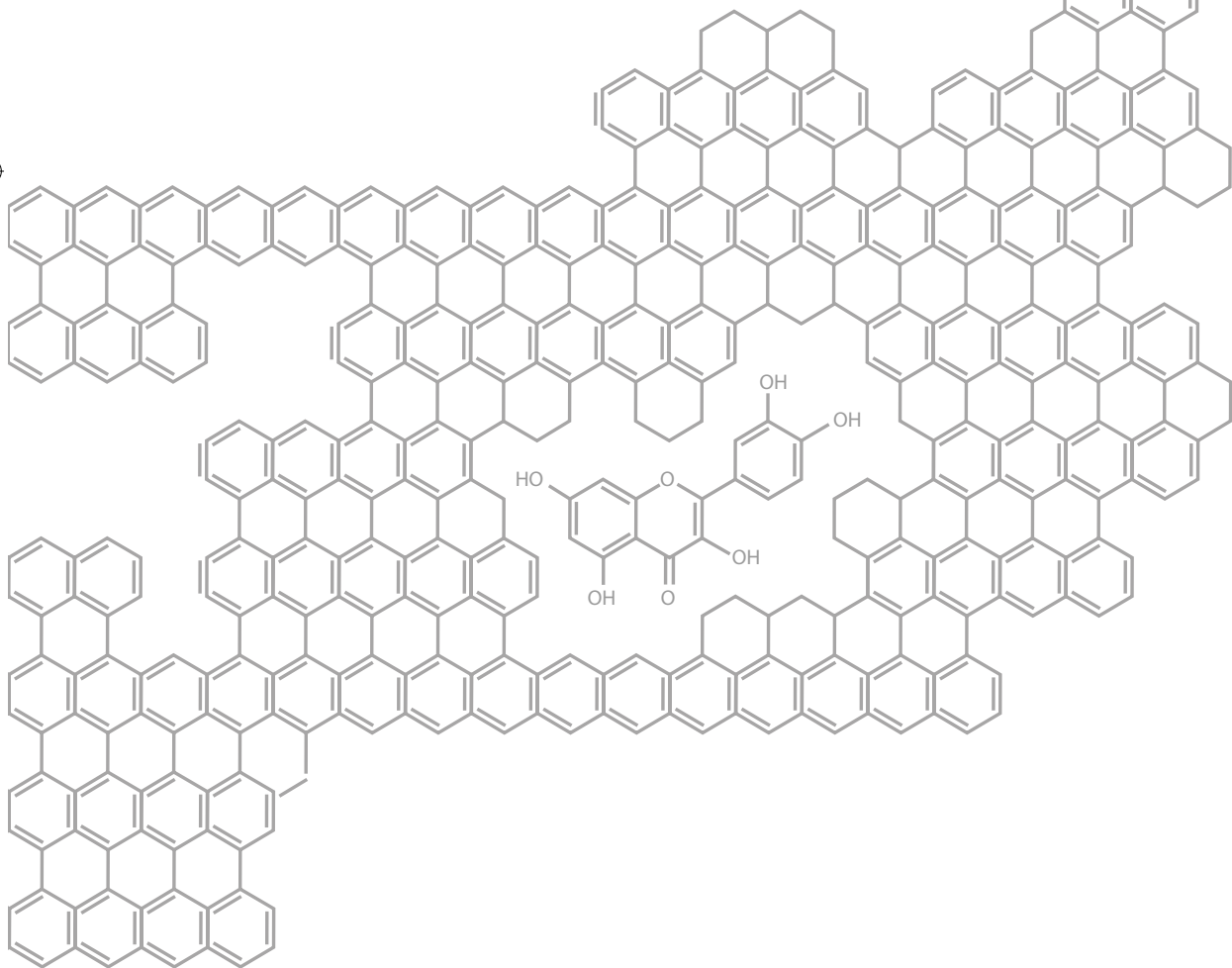
Although the induction of EpRE-mediated gene expression is an important factor why flavonoids are health-promoting, it is not likely that flavonoids exert their beneficial effects through only one mechanism. It is more likely that these food compounds can trigger multiple, parallel or cascade mechanisms. Multiple gene expression analysis of quercetin in colon cancer cells *in vitro* showed differential expression of genes involved in tumor suppression, cell adhesion, transcription and signal transduction, contributing to the anticarcinogenic potential of flavonoids (van Erk *et al.* 2005). Quercetin can downregulate many cell cycle genes, resulting in a decrease in cell proliferation (van Erk *et al.* 2005) and up-regulate fatty acid catabolism pathways, resulting in a decrease of fatty acid levels (de Boer *et al.* 2006a). Moreover, as shown in this thesis, higher levels of flavonoids (up to 100 μM) can lead to modified expression of other genes and pathways, like the increased expression of XRE-regulated genes like phase 1 enzymes.

Low concentrations of flavonoids (1-20 μM) lead to the activation of only phase 2 enzymes, while higher concentrations (20-100 μM) trigger the activation of phase 1 enzymes and may inhibit NQO1 (**Chapter 3, 4**). Therefore, it is questionable, if increased flavonoid consumption will lead to a further improvement of the human health status to a level above that which is already achieved by normal dietary levels of flavonoid intake. Although concentrations higher than the physiological level of flavonoids (maximum of 10 μM) are found in the present studies to be undesirable and can possibly lead to induction of phase 1 enzymes and NQO1 inhibition, the question remains, if increased flavonoid consumption will actually lead to increased flavonoids levels in the human body. Accumulating evidence indicates that flavonoids are poorly bioavailable and reach only low, micromolar concentrations in human plasma, even after the intake of large amounts of flavonoid-rich foods (Manach *et al.* 2005). Therefore, flavonoids levels up to 100 μM are not likely to occur with the exception of the intestinal levels that might be expected upon oral supplement intake. Especially for this organ, further studies are needed on the molecular mechanisms of action and metabolism of flavonoids in the human body to be able to define the optimal concentration of flavonoid intake to increase its beneficial effects, without triggering undesired side effects like phase 1 induction and NQO1 inhibition.





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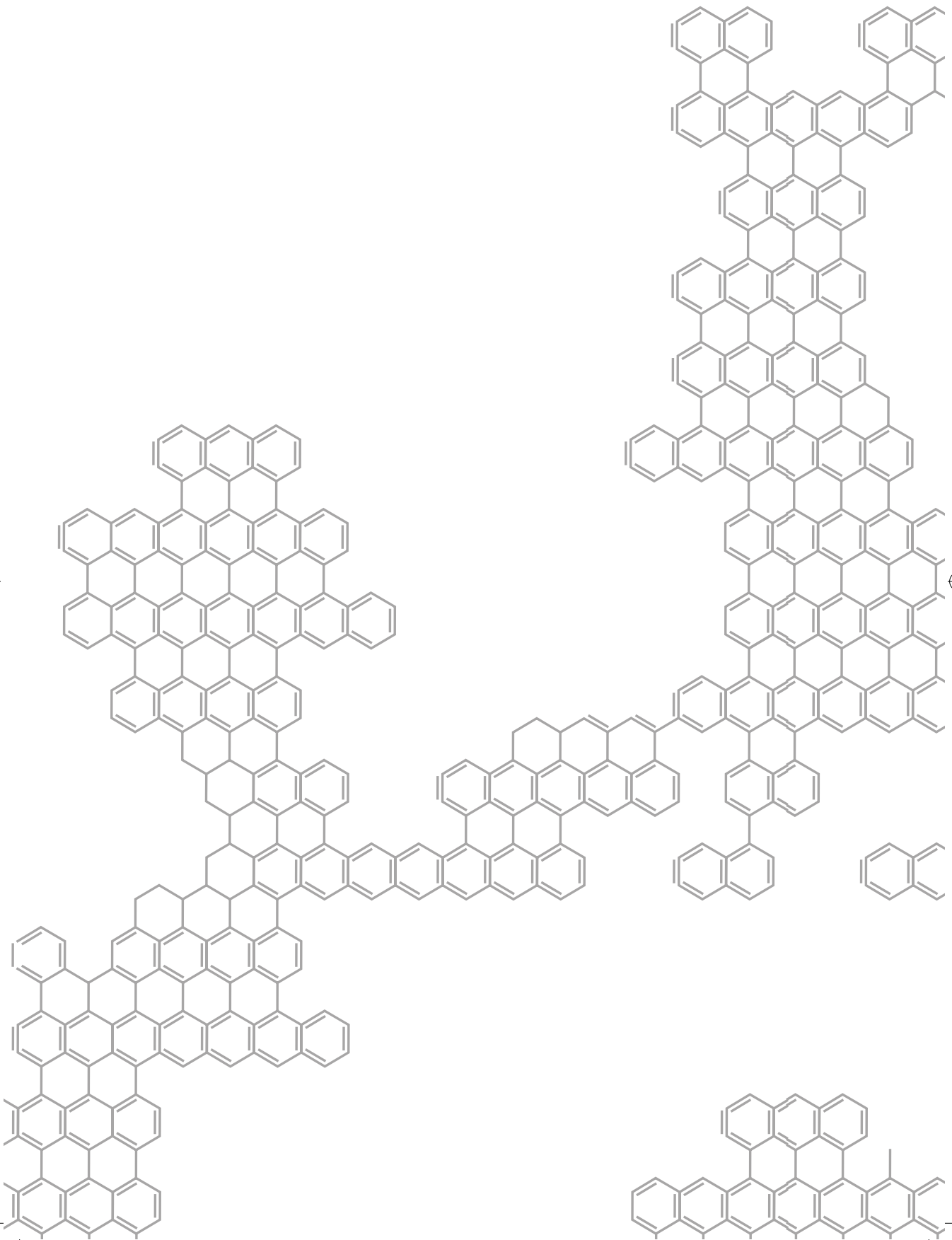
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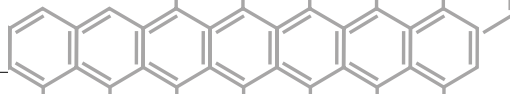
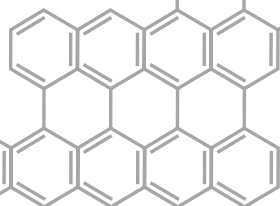
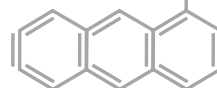
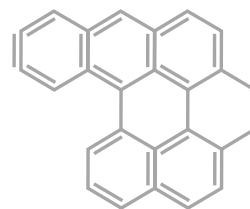
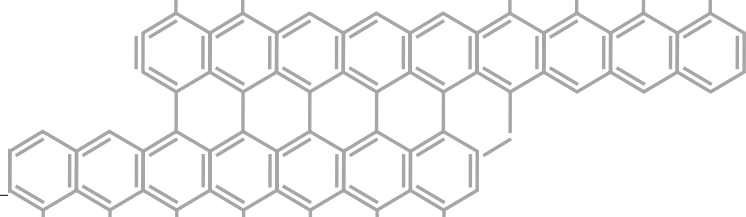
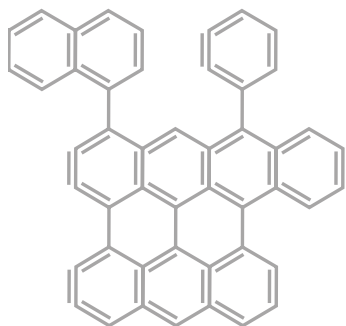
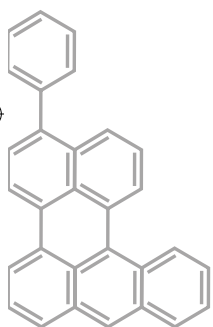
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Samenvatting

Zusammenfassung



Samenvatting

Dit proefschrift beschrijft de *in vitro* en *in vivo* wisselwerking tussen NAD(P)H: quinoneoxidoreductase 1 en gezondheidsbevorderende flavonoïden. Flavonoïden zijn belangrijke bestanddelen in groenten en fruit waarvan wordt gedacht dat ze gezondheidsbeschermende werking hebben. Een van de mogelijke mechanismen voorgesteld voor het optreden van het beschermende effect van deze bioactieve bestanddelen is de verhoging van de werking van enzymsystemen die verantwoordelijk zijn voor de inactivatie van chemische carcinogenen, zoals NAD(P)H: quinone oxido reductase 1 (NQO1). Inductie van NQO1-activiteit wordt beschouwd als een biomarker voor anticarcinogene chemopreventieve werking, omdat is gevonden dat NQO1 betrokken is bij de bescherming tegen bepaalde toxische stoffen en reactieve vormen van zuurstof (Ross en Siegel 2004). Tussen NQO1 en flavonoïden, waarvan wordt aangenomen dat zij ieder op zich gezondheidsbeschermende factoren zijn, bestaat een complexe wisselwerking. Enerzijds werken een aantal flavonoïden als NQO1-inducers door het expressieniveau van het *NQO1* gen te verhogen via het electrophile-responsive element (EpRE). Anderzijds zijn bepaalde flavonoïden effectieve remmers van de NQO1 enzymactiviteit *in vitro*. Het doel van dit proefschrift is de complexe wisselwerking tussen flavonoïden en NQO1 op te helderen.

Allereerst werd de remming van NQO1 door flavonoïden bestudeerd, hetgeen zou duiden op een mechanisme dat de gunstige eigenschappen van deze natuurlijke bestanddelen juist zou tegenwerken (**Hoofdstuk 2 en 3**). Inhibitiestudies met gezuiverd NQO1 en moleculaire docking werden uitgevoerd om een model te beschrijven voor de remming van NQO1 door flavonoïden (**Hoofdstuk 2**). De remming van NQO1 door flavonoïden bleek competitief ten opzichte van NADH. Ofschoon *in vitro* studies met gezuiverd NQO1-eiwit gedetailleerd inzicht geven in het mechanisme van enzymremming, kunnen deze niet de metabole condities in de intacte cel nabootsen, omdat ze geen rekening houden met van nature aanwezige concentraties van cofactoren en mogelijke remmers. Daarom werd een methode ontwikkeld om de NQO1-activiteit in levende cellen te bepalen (**Hoofdstuk 3**). Deze studies toonden aan dat, hoewel flavonoïden potentieel een remmende werking hebben op de NQO1-activiteit *in vitro*, de situatie in levende cellen vele malen complexer is. Uit het feit dat de remming van NQO1 door flavonoïden wordt veroorzaakt door competitieve remming ten opzichte van NAD(P)H, volgt dat de remming van NQO1 in intacte cellen afhangt van de intracellulaire NAD(P)H-concentratie in deze cellen. Onze studies laten zien dat bij orale inname van flavonoïden de remming van NQO1 door flavonoïden waarschijnlijk niet van fysiologische relevantie is en daarom niet in strijd is met de gerapporteerde gunstige effecten van deze fytochemicaliën.

De studies in dit proefschrift tonen duidelijk aan dat de belangrijkste factor in de wisselwerking tussen NQO1 en flavonoïden niet de remming van NQO1 door flavonoïden is, maar de door flavonoïden veroorzaakte inductie van de NQO1 gen-expressie.

Flavonoïden bleken gezondheidsbeschermende gen-expressie te induceren (**Hoofdstuk 4, 5, 6, 7**) via het electrophile-response element (EpRE), een regulerende nucleotidensequentie welke verantwoordelijk is voor de gecoördineerde activering van de transcriptie van genen betrokken bij fase 2 biotransformatie, zoals NQO1. Cellulaire reporter-genstudies hebben duidelijk gemaakt dat het niet waarschijnlijk is dat upstream XRE-gemedieerde gen-expressie nodig is om EpRE-gemedieerde gen-expressie te bewerkstelligen (**Hoofdstuk 4**). Alle onderzochte flavonoïden bleken EpRE- en XRE-gemedieerde gen-expressie te induceren, zij het in een verschillende concentratiegebied. Bij fysiologisch relevante concentraties (1-10 μM) is de inductie van gen-expressie via het EpRE transcriptie-enhancer element overheersend en leidt vooral tot verhoogde niveaus van ontgiftende fase-2-enzymen. Niettemin kunnen hogere concentraties flavonoïden (tot 100 μM) leiden tot een toename van de expressie van door een XRE gereguleerde genen zoals fase-1-enzymen, zoals beschreven in **Hoofdstuk 4**. Kwantummechanische berekeningen lieten zien dat flavonoïden met een hogere intrinsieke potentie om oxidatieve stress en redox cycling te veroorzaken, de meest potente inducers van NQO1 zijn (**Hoofdstuk 5**). Het is intrigerend en in strijd met de algemeen geldende opvatting, dat de pro-oxidante werking van flavonoïden een gunstige respons kan opwekken, in dit geval de EpRE-gemedieerde gen-transcriptie van ontgiftende enzymen, wijzend op een gunstig effect van een negatief veronderstelde reactie. Verdere ondersteuning voor de rol van de als gevolg van de pro-oxidante werking van flavonoïden gegenereerde flavonoïd chinon metabolieten bij de activering van EpRE-gemedieerde gen-expressie wordt gepresenteerd in **Hoofdstuk 6**. Radioactieve bindingstudies toonden modificatie aan van Keap1 door de chinon vorm van het flavonoïde quercetin en maakten duidelijk dat de activering van EpRE-gemedieerde gen-expressie door quercetin te danken is aan thiol-adductvorming door quercetine chinon aan Keap1, hetgeen leidt tot Keap1-polymerisatie en afname van het post-transcriptionele niveau van het repressoreiwit Nrf2. Ook werd de potentie bestudeerd van *in vivo* metabolieten van quercetin om de door EpRE gemedieerde gen-expressie te induceren (**Hoofdstuk 7**). De resultaten tonen aan dat, hoewel de glucuronides van quercetine de belangrijkste systemische metabolieten zijn in de circulatie, gedeglucuronideerde derivaten de actieve bestanddelen zijn die verantwoordelijk zijn voor de gunstige EpRE-gemedieerde gen-expressie-effecten in intacte cellen.

Samenvattend kan worden gezegd dat het onderzoek beschreven in dit proefschrift inzicht geeft in de complexe wisselwerking tussen NQO1 en flavonoïden op eiwit- en gen-expressieniveau. Geconcludeerd kan worden dat het chemopreventieve effect van inductie van EpRE-gemedieerde gen-transcriptie door flavonoïden het meest dominant is bij fysiologisch relevante concentraties. Lage concentraties van flavonoïden (1-20 μM) leiden tot de activering van alleen fase-2-enzymen, terwijl hogere concentraties (20-100 μM) ook de activering bewerkstelligen van fase-1-enzymen en daarnaast ook de enzymatische activiteit van NQO1 zouden kunnen remmen.

Zusammenfassung

Diese Doktorarbeit beschreibt das *in vitro* und *in vivo* Zusammenspiel zwischen NAD(P)H: quinone oxidoreductase 1 und gesundheitsfördernden Flavonoiden. Flavonoide sind sekundäre Pflanzeninhaltsstoffe. Sie kommen in allen Obst- und Gemüsesorten vor und werden als gesundheitsfördernd diskutiert. Flavonoide als sogenannte bioaktive Inhaltsstoffe sind in der Lage, die Aktivität von spezifischen Enzymen, die für die Deaktivierung von karzinogenen Substanzen verantwortlich sind, zu regulieren. Zu diesen Enzymen gehört zum Beispiel das Enzym NAD(P)H: quinone oxidoreductase (NQO1). NQO1 spielt im Schutz gegen toxischen Substanzen und reaktive Formen des Sauerstoffes eine wichtige Rolle, indem es an deren Metabolisierung beteiligt ist (Ross and Siegel 2004). Die Erhöhung der NQO1 Enzymaktivität, beispielsweise durch bioaktive Substanzen, wird daher als Biomarker für antikarzinogene und chemopräventive Wirkung angesehen. NQO1 und Flavonoide werden beide als gesundheitsfördernde Faktoren angesehen. Das Zusammenspiel und die Wechselwirkungen der beiden Faktoren sind jedoch komplex und kontrovers. Von einigen Flavonoiden ist bekannt, dass sie die Gen Expression von NQO1 induzieren, indem sie das elektrophile Response-Element (EpRE) aktivieren. Andere Studien belgen, dass einzelne Flavonoide effiziente Inhibitoren der NQO1-Aktivität *in vitro* sind. Im Rahmen dieser Doktorarbeit sollte daher das komplexe Zusammenspiel zwischen Flavonoiden und NQO1 in Hinblick auf die Induktion der Gen Expression und der Enzyminhibition erforscht werden.

Als erstes wurde die Inhibition der NQO1-Aktivität durch Flavonoide untersucht. Die nachgewiesene Inhibition spricht gegen eine gesundheitsfördernde Wirkung dieser sekundären Pflanzeninhaltsstoffe (**Kapitel 2 und 3**). Die Inhibition von NQO1 durch Flavonoide wurde anhand des isolierten Enzyms studiert. Desweiteren wurde anhand einer molekularen Bindungsstudie ein Model für die Hemmung von NQO1 durch Flavonoide entwickelt (**Kapitel 2**). Das Ergebnis dieser Studie zeigt, dass die Inhibition von NQO1 durch Flavonoide kompetetif gegen NADH ist. *In vitro* Studien mit isolierten Enzymen verschaffen einen detaillierten Einblick über die mögliche Inhibition von Enzymen. Leider berücksichtigen diese Studien nicht die Konditionen und die Verfügbarkeit einzelner Substanzen in intakten Zellen. Die Präsenz von Cofaktoren und die Verfügbarkeit der Inhibitoren sind wichtige Faktoren für die Inhibition von Enzymen *in vivo* und können bei Studien mit isolierten Enzymen nicht oder nur zum Teil berücksichtigt werden. Um möglichst viele Faktoren bei der Inhibition von NQO1 durch Flavonoide zu berücksichtigen, wurde als Teil dieser Doktorarbeit eine Methode entwickelt, bei der die NQO1-Aktivität in lebenden Zellen über einen längeren Zeitraum beobachtet werden kann (**Kapitel 3**). Die mit Hilfe der neu entwickelten Methode erhaltenen Ergebnisse zeigten, dass Flavonoide zwar NQO1 inhibieren können, die Inhibition in lebenden Zellen jedoch sehr komplex ist und von vielen Faktoren abhängt. Einer dieser Faktoren ist die NAD(P)H Konzentration in den Zellen. Die Inhibition von NQO1 durch Flavonoide ist kompetetif zu NAD(P)H, weshalb eine natürlich hohe NAD(P)H-Konzentration in den Zellen einer

Inhibierung durch Flavonoide entgegenwirkt. Desweiteren zeigten die Ergebnisse dieser Studie, dass bei einer durchschnittlichen Aufnahme von Flavonoiden durch die Nahrung eine Inhibierung von NQO1 unwahrscheinlich ist.

Im weitem Verlauf dieser Doktorarbeit wurde deutlich, dass der wichtigste Aspekt im Zusammenspiel zwischen Flavonoiden und NQO1 nicht die Inaktivierung des Enzyms, sondern die Aktivierung der Gen Expression und somit der Produktion des Enzyms ist. **Kapitel 4, 5, 6 und 7** befassen sich mit der Erhöhung der NQO1-Gen Expression durch Flavonoide über das elektrophile Response Element (EpRE). Das EpRE ist eine regulierende Gensequenz, die in die koordinierte transkriptomale Aktivierung der Gene, die für die Phase 2-Biotransformation zuständig sind, involviert ist. Das schließt auch NQO1 ein. Im Rahmen dieser Doktorarbeit wurden Zellstudien durchgeführt, welche belegen, dass die Gen Expression durch das xenobiotische Response-Element (XRE), welches dem EpRE voran liegt, nicht nötig ist, um eine EpRE gesteuerte Gen Expression zu bewirken (**Kapitel 4**). Allerdings sind Flavonoide in bestimmten Konzentrationen in der Lage, sowohl EpRE als auch XRE gesteuerte Gen Expression zu induzieren. Bei physiologisch relevanten Konzentrationen (1-10 μM) ist die Induktion der Gen Expression über das EpRE, welches für die Regulierung der Phase 2-Enzyme verantwortlich ist, dominant. Die Induktion über das XRE, welches für die Regulierung der Phase 1-Enzyme verantwortlich ist, erfolgt hauptsächlich in höheren Konzentrationen (bis zu 100 μM).

Quantum mechanische Kalkulationen ergaben, dass Flavonoide mit einem hohen inneren Potential, oxidativen Stress und Redoxzirkulation zu bewirken, am stärksten die NQO1-Gen Expression induzieren (**Kapitel 5**). Die im Rahmen dieser Doktorarbeit erhaltenen Ergebnisse bewiesen, dass die prooxidativen Eigenschaften der Flavonoide für den gesundheitsfördernden Effekt, nämlich die Induzierung der antikanzinogenen Enzyme, verantwortlich ist. Dies steht im Gegensatz zu der allgemeinen Annahme, dass die prooxidative Eigenschaft von Flavonoiden eine toxische Wirkung besitzt. **Kapitel 6** beschreibt die Aktivierung der EpRE-gesteuerten Gen Expression durch die prooxidative Wirkung von Flavonoiden. Bindungsstudien mit radioaktiv markierten Flavonoiden zeigten, dass Flavonoide, in diesen Falle Quercetin, die EpRE-gesteuerten Gen Expression durch eine Modifizierung des Keap1-Proteins aktivieren. Keap1 ist im normalen Zustand für die Inhibierung der EpRE-gesteuerten Gen Expression verantwortlich und wird durch die Bindung mit Quercetin polymerisiert. Durch diese Polymerisation wird das posttranskriptionale Keap1 reduziert, und die nötigen Faktoren für die EpRE gesteuerten Gen Expression werden freigesetzt. Desweiteren befasst sich diese Doktorarbeit mit der Induzierung von EpRE-gesteuerten Gen Expression durch in vivo Metabolite von Quercetin (**Kapitel 7**). Quercetin wird in der glucuronierten Form im Blutkreislauf transportiert. Diese Glucuronide von Quercetin sind in der Lage, die Gen Expression von NQO1 zu induzieren. Allerdings werden die Glucuronide in der Zelle wieder in Quercetin und Glucuronsäure gespalten, so dass dennoch das Quercetinaglycon die aktive Komponente ist, die für die gesundheitsfördernde, EpRE-gesteuerter Gen Expression verantwortlich ist.



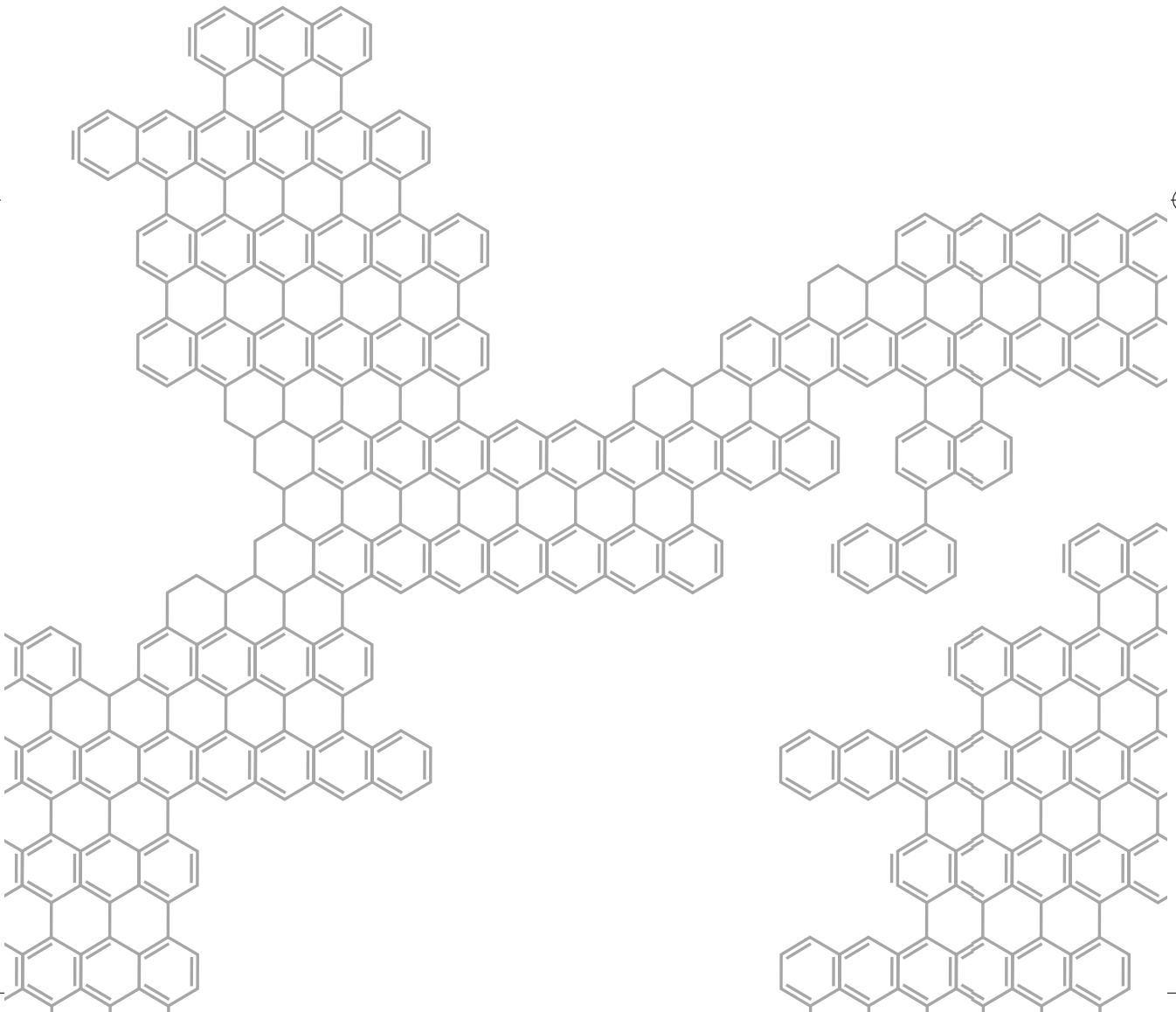
Zusammenfassung

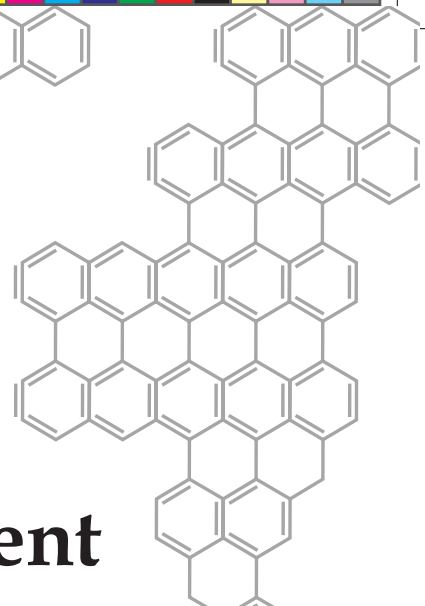
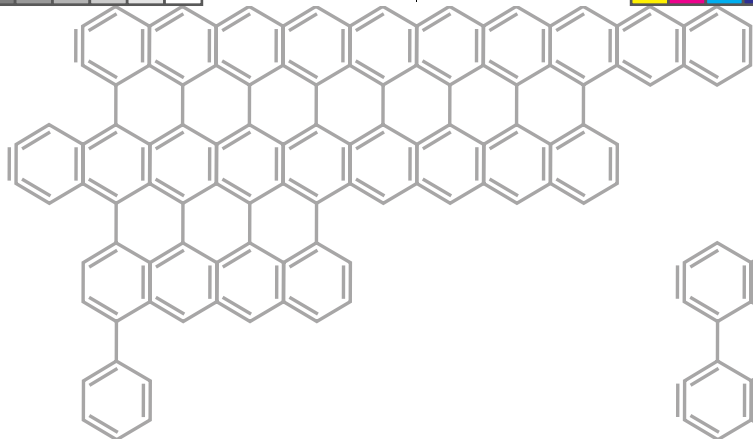
Zusammenfassend gibt diese Doktorarbeit einen Einblick in das komplizierte Zusammenspiel zwischen NQO1 und Flavonoiden auf Protein und Gen Expressions Ebene. Hierbei wurde gezeigt, dass die Induzierung der EpRE gesteuerter Gen Expression in den physiologisch relevanten Konzentrationen dominant ist. Niedrige Konzentrationen (1-20 μM) bewirken die Aktivierung von Phase 2- Enzymen wie NQO1. Höhere Konzentrationen (20 - 100 μM) aktivieren Phase 1-Enzyme und inhibieren die NQO1 Enzymeaktivität.



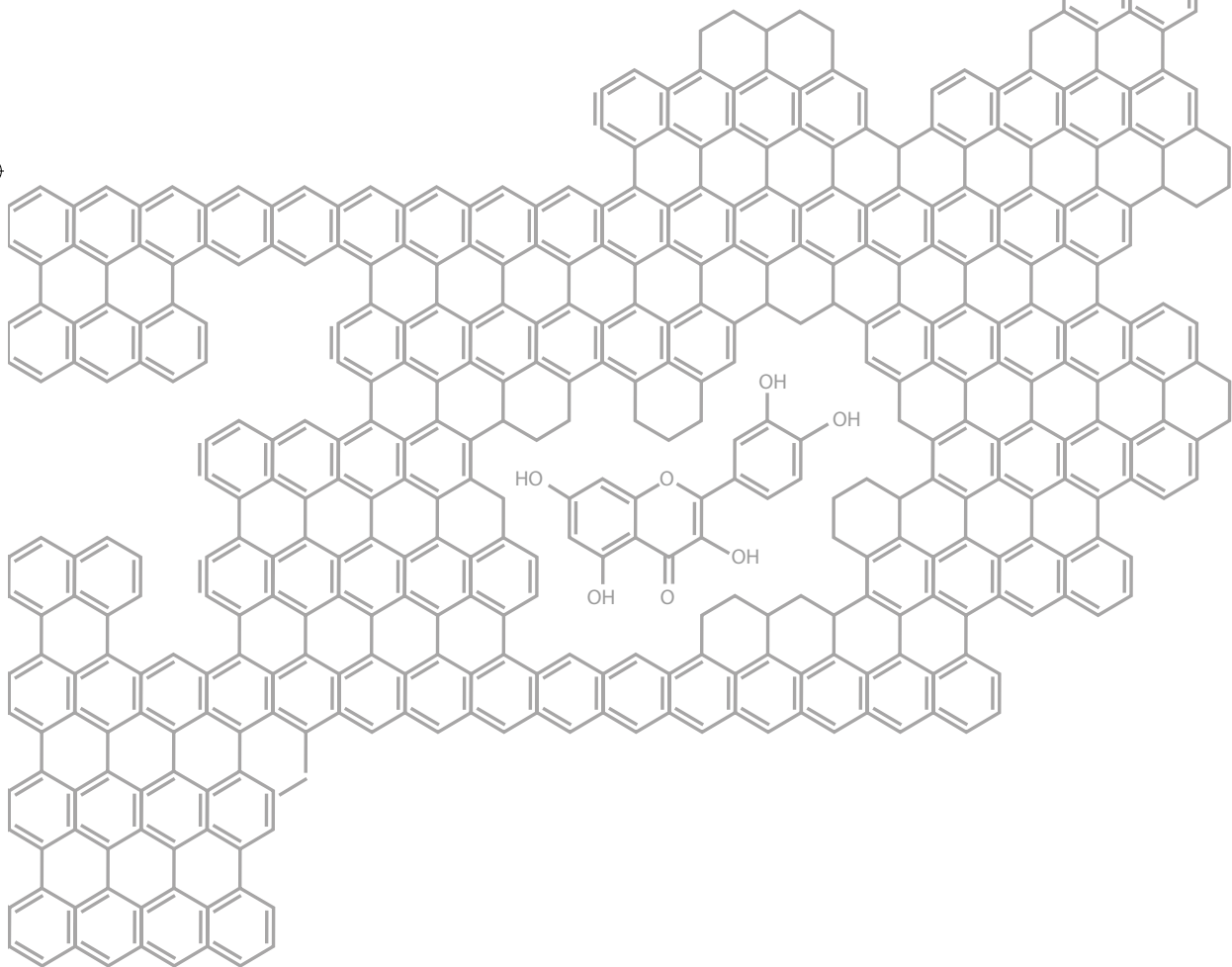
Zusammenfassung







Acknowledgement





Acknowledgement

Four and half years ago I came to Wageningen for an interview for a PhD project. It was mid march, right after my final exams in Germany. I applied for the vacancy in a very hectic period of life, and somehow managed to loose all information about the project I had in the meantime. Therefore I arrived at the interview without even knowing the title of the project. Well, today I know, it was called "*In vitro* and *in vivo* interplay between NAD(P)H: quinine oxidoreductase 1 and flavonoids", the PhD project I started 4 years ago.

I will never forget this interview and although I was so unprepared, the three people present that day gave me the position and made this thesis here possible. Therefore, my special thanks to those 3, my two supervisors Willem van Berkel and Jac Aarts and my promoter Ivonne Rietjens for giving me the chance to do my PhD at the departments of Biochemistry and Toxicology. All three had great input in my thesis. Dear Ivonne, thank you for the guidance and the fruitful discussions throughout the 3 years. I learned a lot from you, your efficiency, your strength and self confidence. I grew in the last 4 years and you influenced this in many ways. Thank you very much! Dear Willem, it has been a pleasure to work with you! You are a great supervisor. You gave me freedom to find my own way, but never left me lost in the big world of science. Although you preferred staying in the background and let me explore, you had great input in the direction and the outcome of my thesis. And you, dear Jac, thank you so much for the last 4 years. It was fun to stand with you in the lab and pick clones. You taught me a lot in the molecular field and I will never forget that you cannot "induce the EpRE". I also would like to thank my second promoter Sacco de Vries. Thank you for the hints you gave me in colloquia and for being the head of such a nice department of Biochemistry.

I spent my PhD time in 2 departments, exactly 50:50. My computer was at the Biochemistry, while my pipettes and labspace was at Toxicology. That helped me to integrate in both departments to 100% and I felt home at both of them. Thanks to all my nice colleagues in both departments for their scientific help and support. All of you contributed to my thesis in some way and helped me surviving my PhD dips. Special thanks to Hans (TOX), Hans (BIC), Marella, Bert, Willy, Jan-Willem for helping me finding my way through the labs and beyond. Thanks to Walter & Cathy for their support in dealing with a bunch of questioning enzymology students. Laura (BIC), Irene & Gré, you are defiantly the most important administrative persons in the departments. Thanks to Ans Soffers, Tinka, Jacques & Carlo for their scientific guidance and discussions and Gerrit for finding two wonderful students for me. Thanks to Letty & Bas for guiding me through my POT courses. Laura (TOX), you have been a great help to me throughout my PhD time. Thank you for taking care of my cells and students during weekends and holidays. Anne-Marie Gerritsen bedankt voor jou hulp en het wassen van al mijn vies pipetten op Tox.

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And now, the long list of PhD friends. Walter, Ashwin, Pim, Vincent, Vincent, Ans, Wiratno, Elton, Jelmer, Merijn, Marcel, Anne-Marie, Marlous, Ruchira, Sanne, Sofia, Mark K., Mark H., Jose, Maarten, Yves, Niek, Kees-Jan, Eike, Ana & Rumyana thanks for sharing the PhD time with me. It helped to see that I was not the only one having dips and days of frustration. Without you guys, I would not have survived! I enjoyed all the BBQs, PhD trips, dinners and parties with you. Hope we will stay in touch!

Thanks girls, Maaïke, Hester & Suzanne, for all the nice dinners, chats and closed door discussions. Special thanks to the two good friends I made in Wageningen, Gabriele & Sangita. True friends are hard to find and I am glad that I found you!

My thesis would have been much thinner, without the help of Sovianne & Maria, my two fantastic students! I had a very good time with you two and thank you so much for your contribution to chapter 4 and 7.

Vielen Dank Mark für das tolle Cover!

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Ohne viele Worte: Danke Hauke, dass Du immer für mich da bist!



About the Author

Curriculum vitae

Yuen Yee Hilz (née Lee) was born on May 25, 1977, in Kuala Lumpur, Malaysia. In autumn 1979 she immigrated together with her family to Germany. In 1996 she finished her pre-university education (Gymnasium Winsen, Germany) and started her studies of Food Chemistry at the University of Hamburg (Germany) in the same year. After her BSc study, she spent two terms at the University of Reading (United Kingdom) and worked on a research project entitled "Sensory and Analytical Studies of Sweetness Perception" with Prof. Gordon Birch. During her MSc study at the University of Hamburg (Germany) she wrote her diploma thesis on the "Determination of Steroid Hormones in Muscle and Fat of Cattle", by using GC-MS techniques. She obtained her Diploma in Food Chemistry together with the 1st State Examination in Food Chemistry, Microbiology, Botany, and Toxicology in September 2001. Afterwards Yee went as a visiting scientist to the United States Department of Agriculture (USA). There she worked on a research project entitled "Determination of Seleno Amino Acids in Dietary Supplements and Food Products" under the supervision of Dr. Wayne Wolf at the Beltsville Human Nutrition Research Center, Agricultural Research Center, Food Composition Laboratory (USA). From 2002 until 2003, she worked at the Governmental Food Inspection Laboratory, Landesuntersuchungsämter Schleswig-Holstein (Germany), with education in writing expertises based on the German and European food law. In March 2003 Yee obtained the title "Staatlich geprüfter Lebensmittelchemiker" (certified Food Chemist), a protected title for governmental recognised food chemists (more information on http://www.gdch.de/strukturen/fg/lm/beruf_e.htm). From July 2003 until July 2007 she was appointed as PhD student at the Wageningen University (section of Toxicology and laboratory of Biochemistry). She performed her research on the project entitled "*In vitro* and *in vivo* interplay between NAD(P)H: quinone oxidoreductase 1 and flavonoids", as described in this thesis. During the PhD project several courses were attended including courses of the Postdoctoral Education in Toxicology (PET) program, resulting in the registration as Toxicologist.

List of publications

Lee, Y.Y., Westphal, A.H., de Haan, L.H.J., Aarts, J.M.M.J.G., Rietjens, I.M.C.M., van Berkel, W.J.H., Human NAD(P)H: quinone oxidoreductase inhibition by flavonoids in living cells, *Free Radical Biology & Medicine*, 39, 257 – 265 (2005).

Boerboom, A.J. F., Vermeulen, M., van der Woude, H., Bremer, B.I., **Lee-Hilz, Y.Y.**, Kampman, E., van Bladeren, P.J., Rietjens, I.M.C.M., Aarts, J.M.J.J.G., Newly constructed stable reporter cell lines for mechanistic studies on electrophile-responsive element-mediated gene expression reveal a role for flavonoid planarity, *Biochem Pharmacol* 72, 217-26 (2006).

Lee-Hilz, Y.Y., Boerboom, A.J.F., Westphal, A.H., van Berkel, W.J.H., Aarts, J.M.M.J.G., Rietjens, I.M.C.M., Pro-oxidant activity of flavonoids induces EpRE-mediated gene expression, *Chemical Research in Toxicology*, 19, 1499-1505 (2006).

François, K.J., **Lee-Hilz, Y.Y.**, van der Kamp, M., van Heusden, H., Rietjens, I.M.C.M., Vervoort, J., A structural model for the inhibition of NAD(P)H: quinone oxidoreductase 1 by flavonoids, submitted

Lee-Hilz, Y.Y., ter Borg, S., van Berkel, W.J.H., Rietjens, I.M.C.M., Aarts, J.M.M.J.G., Concentration-dependent switch from EpRE- to XRE-mediated gene induction of biotransformation enzymes, submitted

Lee-Hilz, Y.Y., Boeren, S., Westphal, A.H., de Haan, L.H.J., Aarts, J.M.M.J.G., Rietjens, I.M.C.M., van Berkel, W.J.H., Quercetin covalently modifies and inactivates Keap1, a repressor of transcription factor Nrf2, submitted

Lee-Hilz, Y.Y., Stolaki, M., van Berkel, W.J.H., Aarts, J.M.M.J.G., Rietjens, I.M.C.M., Activation of EpRE-mediated gene transcription by quercetin glucuronides depends on their deconjugation, submitted



Awards

Mechanism of flavonoid induced EpRE-mediated gene transcription, 49th annual Protein meeting, Lunteren, The Netherlands (12.-13.12.2005) *Award for best poster*

Activation of cancer protective enzymes by flavonoids, Netherlands Society of Toxicology, Wageningen, The Netherlands (13.-14.06.2006) *Award for best presentation*

Assessment of the health-promoting activity of flavonoids for the application in food, 3rd international conference on biocatalysis in the food and drink industrie, Wageningen, The Netherlands (24.-26.09.2006) *Award for best poster*

Training and supervision plan

Courses

| | | | | |
|-------------------------------|------------|---------|-------|------|
| Food Toxicology & Food Safety | Wageningen | 12-16 | May | 2003 |
| VLAG PhD week | Bilthoven | 22-27 | Sep | 2003 |
| Nutrigenomics | Wageningen | 06-07 | Oct | 2003 |
| Molecular Toxicology | Wageningen | 05-08 | April | 2004 |
| Epidemiology | Utrecht | 23-27 | Aug | 2004 |
| Risk Assessment | Wageningen | 11-22 | Oct | 2004 |
| GFP and LUC | Wageningen | 11-12 | April | 2005 |
| Laboratory Animal Science | Utrecht | 13-24 | Aug | 2005 |
| Ecotoxicology | Wageningen | 22Aug-3 | Sep | 2005 |
| Pathobiology | Utrecht | 03-07 | Oct | 2005 |

Optional

| | | |
|------------------------|------------|-----------|
| Biochemistry Colloquia | Wageningen | 2004-2007 |
| Toxicology colloquia | Wageningen | 2004-2007 |



Meetings

| | | | | |
|---|------------------|-------|-------|------|
| Munic Chemistry days | Munic, Germany | 08-10 | Oct | 2003 |
| DSM Meeting Biocatalysis | Vaalsbroek | 19-21 | Oct | 2003 |
| Protein Meeting Lunteren | Lunteren | 08-09 | Dec | 2003 |
| Protein Meeting Lunteren | Lunteren | 13-14 | Dec | 2004 |
| International Symposium on Flavins and Flavoproteins | Yokohama, Japan | 17-23 | Apr | 2005 |
| Protein Meeting Lunteren | Lunteren | 12-13 | Dec | 2005 |
| Mosbacher Kolloquium | Mosbach, Germany | 05-08 | April | 2006 |
| NVT Meeting | Wageningen | 13-14 | June | 2006 |
| Biocatalysis in the Food and Drinks Industries | Wageningen | 24-26 | Sep | 2006 |
| Protein Meeting Lunteren | Lunteren | 11-12 | Dec | 2006 |





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