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Influence of quercetin and kaempferol on benzo[a]pyrene-mediated effects via AhR- and Nrf2-pathways in human and rat intestinal cell lines

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Abbreviations

AhR	aryl hydrocarbon receptor
AhRR	aryl hydrocarbon receptor repressor
AiP	AhR interacting protein
Akt	protein kinase B
APS	ammonium peroxodisulfate
ARE	antioxidant response element
ARNT	AhR nuclear translocator
ATP	Adenosine triphosphate
B[a]P	benzo[a]pyrene
BSA	bovine serum albumin
C/EBP- β	CCAAT/enhancer binding protein-β
cAMP	cyclic adenosine monophosphate
cDNA	copy DNA
Cul3	cullin-3
CYP	cytochrome P450
CYP1A1	Cytochrome P450 1A1 isoform
DEPC	diethylpyrocarbonate
DNA	desoxyribonucleic acid
dNTPs	desoxynucleosidtriphosphates
E2	Ubiquitin-conjugating enzyme
EC50	effective concentration with activity reduction of 50%
ECL	enhanced electrochemiluminescence
EDTA	ethylene diamine tetra-acetic acid
EQ	ethoxyquin
EROD	ethoxyresorufin-O-deethylase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GCS	glutamyl-cysteine synthetase
GSH	glutathione
GST	glutathione-S-transferase
h	hours
HIF-1α	hypoxia-inducible factor-1α protein
HO-1	heme oxygenase 1

Hsp90	heat shock protein 90kD
IC50	inhibitor concentration with activity reduction of 50%
JNK	c-Jun N-terminal kinase
K	kaempferol
Keap1	Kelch-like ECH-associated protein 1
LPH	lactase-phlorizin hydrolase
MAPK	mitogen-activaed protein kinase
MMLV	Moloney murine leukemia virus
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
NQO1	NAD(P)H dehydrogenase (quinone) 1
Nrf2	nuclear erythroid 2 p45-related factor 2
p23	chaperone protein 23kD
PAH	polycyclic aromatic hydrocarbon
PBS	phosphate buffered saline
PBST	PBS-Tween
PCR	polymerase chain reaction
PI3K	phosphatidylinositol 3-kinase
PKC	protein kinase C
PXR	pregnane X receptor
Q	quercetin
RNA	ribonucleic acid
RNase	ribonuclease
Roc1	RING finger protein of cullins
ROS	reactive oxygen species
RT	reverse transcription
RT-PCR	reverse-transcription-PCR
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
siRNA	small interfering RNA
sMaf	small MARE-binding components

tBHQ	*tert.*-butylhydroquinone
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TEMED	N,N,N',N'-tetraethylmethylenediamine
Tris	tris-hydroxymethyl-aminomethane
Ub	ubiquitin
UGT	uridine 5'-diphosphate-glucuronosyltransferase
XRE	xenobiotic-response element

1. Introduction

1.1. Gastrointestinal tract

The human gastrointestinal tract (GI tract) is a complex system of organs in mammalian species fulfilling ingestion, digestion, absorption, and defecation as major functions. Morphologically the gastrointestinal tract comprises esophagus, stomach, small and large intestine, and ends with the rectum. Most chemical digestion takes place in the small intestine. The digestive enzymes that are active there are predominantly secreted by the pancreas and enter the small intestine via the hepato-pancreatic duct. Nutrient digestion within the small intestine produces a mixture of monosaccharides, small peptides, amino acids, fatty acids, and monoglycerides. The final digestion and absorption of these substances occurs in the villi which line the inner surface of the small intestine. Incorporated in the plasma membrane of the microvilli are a number of enzymes that complete digestion. That means that the first pass of a nutritional substance takes place in the duodenum. The low molecular weight products of enzymatic digestion are directed into blood or lymph fluid by membrane-bound transport. The small intestine is the site where most of the nutrients from ingested food are absorbed. It is divided into three structural parts: duodenum, jejunum and ileum. Also the large intestine is divided into three parts: cecum, colon and rectum (L.C. Junqueira and J. Carneiro, 1996).

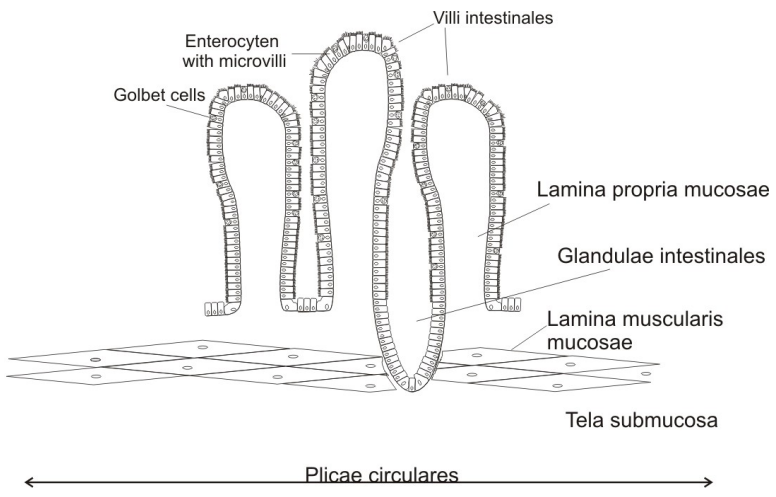


Fig. 1.1. Stucture of intestinal villi. The inner wall of the small intestine is covered by numerous folds of mucous membrane (plicae circulares). The entire luminal surface has villi, small projections of mucosa. The villi are lined with simple columnar epithelial cells (enterocytes). The cells have an apical brush border, also known as microvilli. Both villi and microvilli function to increase the surface area for greater absorption.

Interestingly, cancer of the small intestine is relatively rare compared to other gastrointestinal malignancies such as gastric cancer (stomach cancer) and colorectal cancer. Duodenal cancer

has more in common with stomach cancer, while cancer of the jejunum and ileum has more in common with colorectal cancer (www.who.int/mediacentre/factsheets/fs297/en/).
Since the major site of digestion is localised in the small intestine (90% of the total nutritional substances are absorbed in this region of the gut), the metabolism of pro-carcinogens in duodenal epithelial cells and the modulatory effect of dietary flavonols are in focus for our experiments.

1.2. Polycyclic aromatic hydrocarbons (PAH) are environmental contamination

PAH are formed during the burning of coal, oil, gas, wood, rubbish or other organic substances like tobacco. PAHs are ubiquitous environmental contaminants, and there are some natural sources, such as forest fires and volcanoes. PAHs are also found in foods. Studies have shown that most food intake of PAHs comes from cereals, oils and fats. Human exposure to PAH occurs by intake of contaminated vegetables and by consumption of foods that have been broiled (B.K. Larsson et al., 1983). Benzo[a]pyrene (B[a]P) is a five-ring PAH and is a potent and best investigated carcinogen, acting as initiator and promotor (R.E. Albert et al., 1991). B[a]P was determined in 1933 to be the component of coal tar responsible for the first recognized occupation-associated cancers, the sooty warts (cancers of the scrotum) suffered by chimney sweeps in 18th century England. This carcinogen is a widespread contaminant in the human and animal environment. H.A. Hattermer-Frey et al. (1991) showed that humans are exposed to B[a]P from air, water and food whereas 97 % of the human exposure occurs from the food chain. For example, the ingested weekly dose of B[a]P from charcoal-broiled meat and smokes food has been estimated to vary between 0.01 and 4.0 µg/person (P.L. Lioy et al, 1988). B[a]P is a pro-carcinogen, meaning that it has to be enzymatically activated to the ultimate mutagen, (+)benzo[a]pyrene-7,8 dihydrodiol-9,10 epoxide by a phase I enzyme such as CYP1A1. The diol epoxide covalently binds to DNA as shown in fig. 1.2. K. Alexandrov et al. (1996) was able to detect benzo[a]pyrene diolepoxide-DNA adduct formation in human colon mucosa by means of HPLC analysis. These results indicate that in the duodenum the fast phase II enzyme-mediated detoxification and elimination of B[a]P could cause a lower cancer risk (fig. 1.2.).

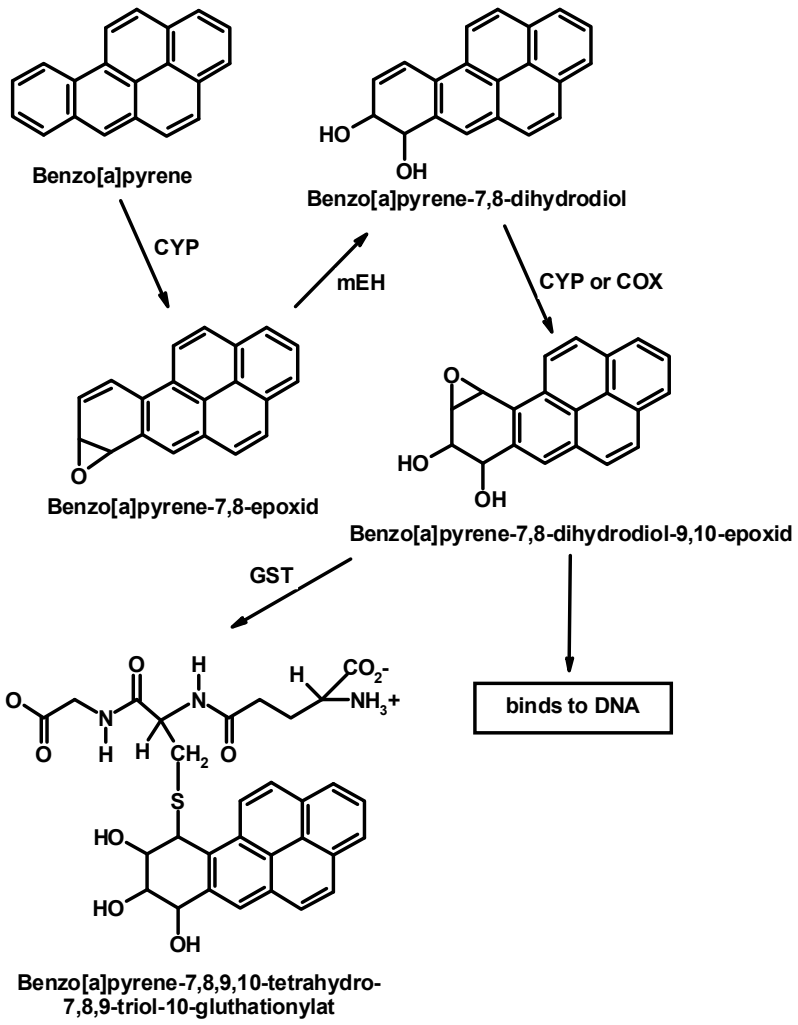


Fig. 1.2. Metabolism and activation of benzo[a]pyrene. B[a]P is initially converted mainly by CYP1A1 or CYP1B1 into the 7,8-epoxide. This epoxide is a substrate of microsomal epoxide hydrolase (mEH), which produces the 7,8-dihydrodiol. Further epoxidation at the vicinal double bond catalysed by CYP1A1, CYP1B1, CYP3A4 or cyclooxygenase (COX) generates the ultimate genotoxic B[a]P-dihydrodiol-epoxid which reacts with nucleophilic sites of DNA to form DNA adducts. Glutathione S-transferase (GST) catalyse the formation of glutathione conjugates and enhances elimination of conjugated species from the cell.

1.3. Chemo-prevention by secondary plant components

More than 8,000 phytochemicals have been identified in fruits, vegetables and grains (R.H. Liu et al., 2003; A. Kale et al., 2008; Y.J. Moon et a., 2005). The polyphenolic flavonoids are the most abundant phytochemicals in our diet and provide much of the flavour and colour to fruits and vegetables. Flavonoids are the most important pigments for flower coloration producing yellow or red/blue pigmentation in petals. Those colours are a mean to attract pollinator animals. They also protect plants from attacks by microbes and insects. They are formed from the combination of derivatives synthesised from phenylalanine, delivered via the shikimic acid pathway, and acetic acid. The structure of the flavonoids is based on the flavonoid nucleus which consists of three phenolic rings referred to as the A, B, and C rings (fig.1.3.; J. Kühnau et al., 1976). The benzene ring A is condensed with a six-member ring

(B), which carries a phenyl substituent (C) in the 2-position. Ring B may be a heterocyclic pyran, which yields flavanols (catechins) and anthocyanidins, or pyrone, which yields flavonols, flavones, and flavanones (S. Aisling et al., 2002). Flavonoids are the most abundant polyphenols in our diets. They can be divided into several classes according to the degree of oxidation of ring B and to the position of ring C : flavones, flavonols, isoflavones, anthocyanins, flavanols, anthocyanidins and flavanones.

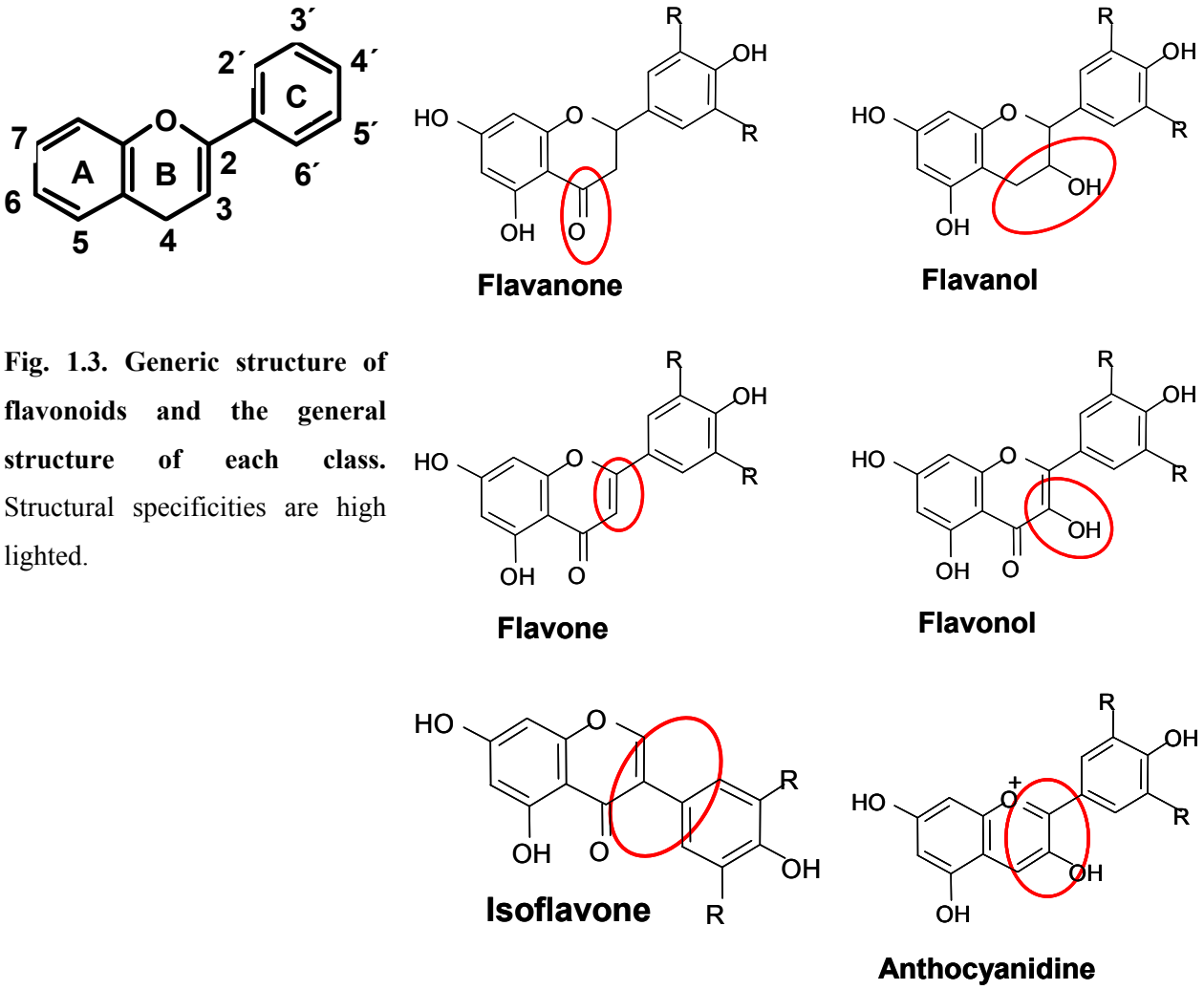
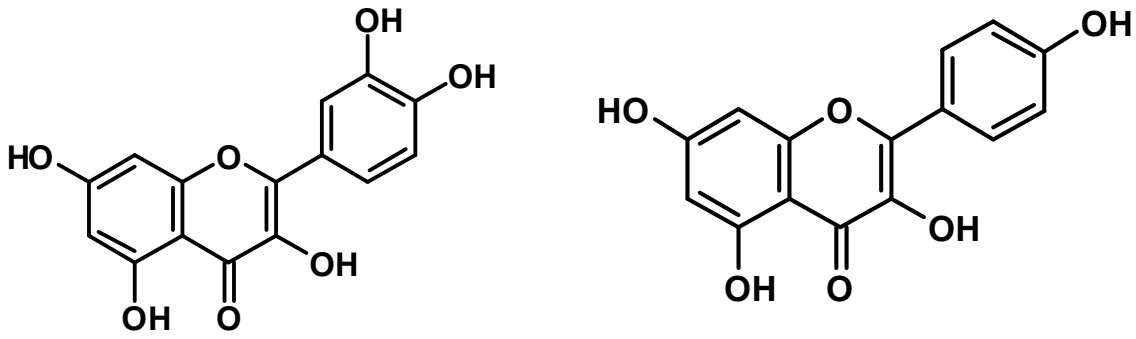


Fig. 1.3. Generic structure of flavonoids and the general structure of each class. Structural specificities are high lighted.

The most abundant flavonoids are the flavonols quercetin and kaempferol, which exist as a variety of glycosides or in their aglycone form. Quercetin occurrs in onion, lovage leave and in paring of many fruits like e.g. apple and red grape. Kaempferol also occurs in cranberries, grapefruit, ginkgo and in grapes. The aglycone forms of quercetin and kaempferol are similar in structure, differing only by one hydroxyl-group in the C-ring (fig 1.4.).



Quercetin **Kaempferol**

Fig. 1.4. The structures of both flavonols quercetin and kaempferol. The aglycone forms of quercetin and kaempferol are similar in structure, differing only by one hydroxy group in the C-ring.

Studies have shown that quercetin and kaempferol are absorbed by the human gut (P.C. Hollman et al., 1997). The flavonol quercetin can be detected in the plasma of non-supplemented humans at concentrations between 0.5 μM - 13 μM (G. Paganga et al., 1997). Quercetin glycosides are cleaved by intestinal microflora before absorption through the colon barrier but intact glycosides can be absorbed in the small intestine (A. Scalbert et al., 2000; P.C. Hollman et al., 1995; P.C. Hollman et al., 1997). After absorption, the aglycone is rapidly conjugated to form glucuronides. There is further evidence that the flavonol glucoronides are deconjugated by β-glucuronidase during cellular absorption in the liver, meaning quercetin and possibly kaempferol aglycones may be taken up at the cellular level. Therefore, *in vitro* addition of quercetin and kaempferol aglycones to human cell lines at levels normally found in plasma after a meal closely mimics the *in vivo* condition (M.L. Ackland et al., 2005). S.P. Boyle et al. (2000) observed that an increased plasma level of quercetin after a meal of onions was accompanied by increased resistance to strand breaks of lymphocyte DNA and by decreased levels of some oxidative metabolites in the urine. Both flavonols are ligands of the aryl hydrocarbon receptor (H.P. Ciolino et al., 1999). Quercetin acts as an antagonist and consequently inhibits other ligands of AhR e.g. B[a]P, and aflatoxin B1 by altering the expression of CYP1A1, 1A2 and 1B1. This inhibition results in reduced B[a]P-DNA adduct formation (Z.C. Kang et al., 1999). Kaempferol also prevents CYP1A1 gene transcription induced by the prototypical AhR ligand, TCDD (H.P. Ciolino et al., 1999). Plant secondary components like flavonoids have biological activity that may be beneficial to health. For instance, these polyphenolic compounds scavenge free radicals and pose antioxidant, anti-thrombotic and anti-carcinogenic activities.

1.4. Aryl hydrocarcon receptor (AhR)-pathway

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor which belongs to the basic-helix-loop-helix (bHLH)/PAS family of heterodimeric transcriptional regulators. The term PAS comes from the first letter of each of the three founding members of the family: PER, ARNT, and SIM. ARNT, the AhR nuclear translocator, was originally identified as a protein that was essential for normal signal transduction by the AhR (E.C. Hoffman et al., 1991). In these proteins, the PAS domain includes two imperfect repetitions of 50 amino acids, PAS-A and PAS-B. It functions as a surface for both homotypic interactions with other PAS proteins and heterotypic interactions with cellular chaperones, such as the 90-kDa heat shock protein (Hsp90). The HLH domains participate in homotypic dimerisation between two bHLH-PAS proteins, and they position the basic regions to allow specific contacts within the major groove of target regulatory elements found in DNA and their transcriptionally active domains are localised within their C-terminal ends (fig.1.5; Z.Y. Gu et al. 2000). The bHLH/PAS proteins are involved in the control of diverse physiological processes such as circadian rhythms, organ development, neurogenesis, metabolism and in the stress response to hypoxia (Z.Y. Gu et al., 2000; R. Barouki et al., 2007).

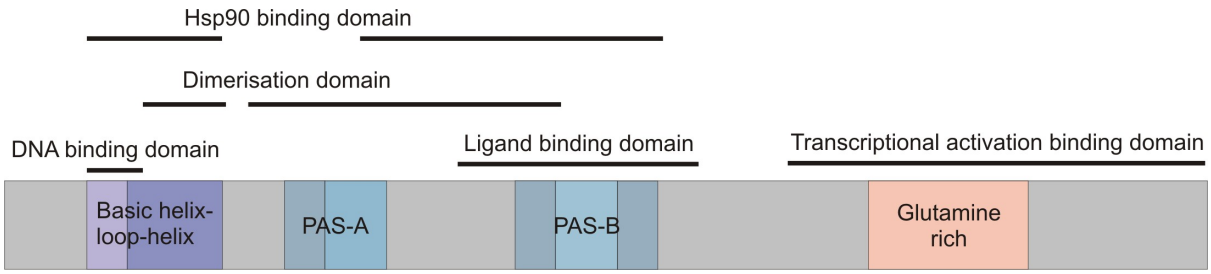


Fig. 1.5. Aryl hydrocarbon receptor functional domains. The location of functional domains is indicated by bars (modified after J. Mimura et al., 2003).

The AhR is known to mediate most of the toxic and carcinogenic effects of a wide variety of environmental contaminants such as PAHs e.g. B[a]P and TCDD. In its non-active form, the AhR is localised in the cytoplasm as a complex with the molecular chaperone Hsp90, the Hsp90-interacting protein p23 and the AhR interacting protein AiP, also known as ARA9 or XAP2 (T. Ikuta et al., 2004). Upon ligand binding, the receptor complex translocates to the nucleus, where the AhR protein dissociates from its chaperone complex and binds to the heterodimer partner ARNT (aryl hydrocarbon nuclear translocator). This heterodimer now binds to a partially characterized set of co-activators and/or co-repressors and the resulting

complex interacts with consensus regulatory sequences (xenobiotic response elements, XREs) located upstream in the promoter of target genes (such as CYP1A1, 1A2, 1B1 and AhRR; see fig. 1.5.). Once transcriptional regulation has occurred, the AhR is exported to the cytosol and degraded by the proteasome.

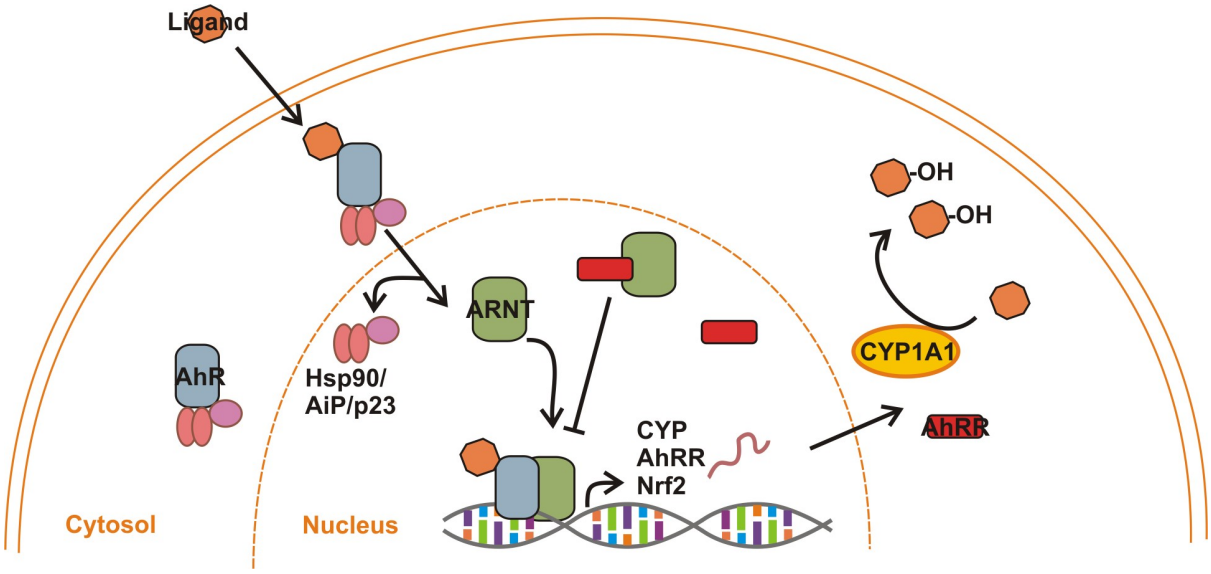


Fig. 1.5. Transcriptional regulation of target genes by the aryl hydrocarbon receptor (AhR). The ligand binds the cytosolic AhR ligand-binding subunit, which undergoes a transformation or activation process involving several steps: translocation in the nucleus; release of a molecular chaperone complex containing at least Hsp90, AiP and p23; and dimerisation with a protein partner, the ARNT. This heterodimer interacts with regulatory DNA sequences (known as xenobiotic-responsive element; XRE) located upstream in the promoter of target genes and stimulate the transcription of genes in the cytochrome P450 CYP1 family CYP1A1 as well as of several Phase II detoxification genes. Also the AhR repressor is a target gene of the AhR. AhRR acts as a negative regulator of AhR signaling by competing with AhR for dimerising with ARNT and binding to the XRE sequence (modified after R. Barouki et al., 2007).

Besides cytochromes P450, the aryl hydrocarbon receptor repressor (AhRR) is also a target gene of the AhR. AhRR acts as a negative regulator of AhR signaling by competing with AhR for dimerising with ARNT and binding to the XRE sequence (J. Mimura et al., 1999). It also contains a DNA-binding (bHLH) and a dimerisation (PAS-A) domain, but lacks PAS-B and Q-rich domains. Similar to ARNT, the AhRR possesses no ligand-binding site (T. Haarmann-Stemmann et al., 2006; T. Baba et al., 2001; Y. Kikuchi et al., 2003; B.R. Evans et al., 2005). Y. Tsuchiya et al. (2003) and K. Gradin et al. (1999) observed that only high cellular levels of AhRR were able to negatively regulate the AhR-pathway; however, lower expression levels

of AhRR do not affect the activated AhR-pathway. Furthermore, it is proposed that either ARNT or the AhRR recruits a corepressor that harbors histone deacetylase activities (T. Haarmann-Stemmann et al., 2006). This indicates that the AhRR plays an important role in the regulation of the AhR-pathway.

The intracellular localization of AhR may be regulated by the masking and unmasking of the nuclear localization signal (NLS) and the leucine-rich nuclear export signal (NES) by some interacting proteins (as shown in fig. 1.6.). The intracellular distribution of nucleo-cytoplasmic shuttling proteins is determined by the balance between nuclear import and export activity. Also phosphorylation or dephosphorylation of the AhR regulates its intracellular distribution, especially at sites close to the NLS or the NES, which results in spatial- and temporal-specific gene regulation mediated by the transcription factors (T. Ikuta et al., 2004; D.A. Jans et al., 1996; E. Le Ferrec et al., 2002). The protein kinase C (PKC) might be essential for both XRE binding and for the transcriptional activation of the CYP1A1 gene (Y.H. Chen et al., 1996; K.W. Bock et al., 2006). PKC-dependent phosphorylation may promote or at least stabilise AhR/ARNT/XRE complex formation, resulting in accelerated transcription.

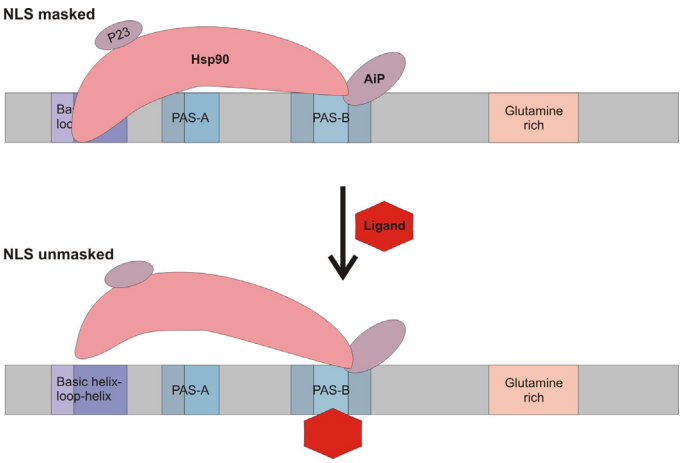


Fig. 1.6. The mechanism of ligand-dependent activation of AhR. The steric hindrance masking of AhR-NLS with two molecules of Hsp90 is essential for cytoplasmic retention of AhR (modified from T. Ikuta et al., 2004).

The high degree of conservation of the Ah-receptor among species, its constitutive pattern of expression during development and in adult tissues and the phenotypic alterations found in mice lacking AhR expression, have provided strong support for the involvement of the AhR in cell physiological functions independent of xenobiotic metabolism e.g. cell proliferation, cells development and differentiation (R. Barouki et al., 2007; P.A. Harper et al., 2006). AhR signaling also includes cross-talk with e.g. a number of protein kinases (as already shown

above) and other signaling-pathways like the Nrf2-pathway (P.A. Harper et al., 2006; A. Puga et al., 2005; C. Köhle et al., 2006; D.B. Carlson et al., 2002).

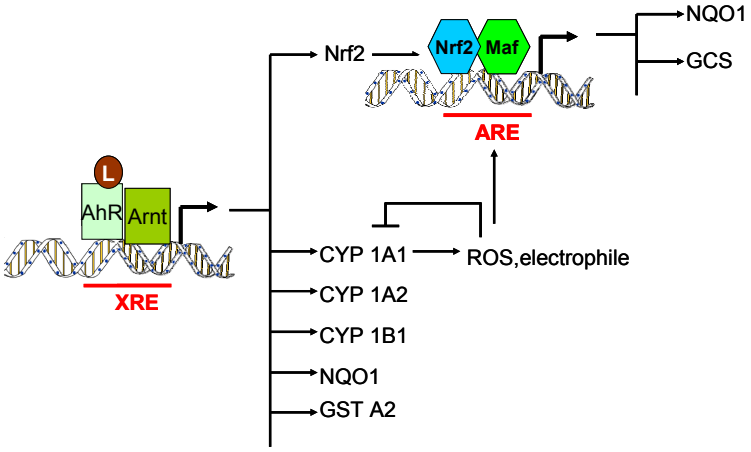


Fig. 1.7. Schematic diagram illustrating Nrf2 as downstream target of the AhR. The schema shows that the AhR-XRE activation induces phase I enzymes and the expression of Nrf2, which in turn activates phase II detoxifying enzymes (modified after W. Miao et al., 2005).

Interestingly, XREs were also identified in the regulatory region of the Nrf2 gene what leads to the assertion that the Nrf2- and AhR- pathways are directly coupled. This cross-talk between these biotransformation systems greatly facilitates detoxification and protection against oxidative/electrophile stress and is intensely investigated in efforts for chemo-protection of cancer and degenerative diseases (fig. 1.7.).

1.5. NF-E2-related factor 2 (Nrf2-)-pathway

NF-E2-related factor 2 (Nrf2) is a member of the CNC (cap'n'collar) transcription factor family (K. Tong et al., 2006). It contains a C-terminal basic leucine zipper structure that facilitates dimerisation and DNA binding (P. Mio et al, 1994). Under basal conditions Nrf2 is bound to Kelch-like ECH-associated protein 1 (Keap1; a sulfhydryl-rich protein which is oxidised by oxidative/electrophile stress) in the cytoplasm due to an interaction between a single Nrf2 protein and a Keap1 dimer (X. Yu et al., 2005). Keap1 serves as a substrate linker protein for interaction of the Cul3-based E3-ubiquitin ligase complex with Nrf2 leading to ubiquitination of Nrf2 and proteosomal degradation (S. Cullinan et al., 2004). Exposure to endogenous activators like reactive oxygen species (ROS), reactive nitrogen species, lipid aldehydes or exogenous agents like heavy metals and electrophilic xenobiotics leads to dissociation of Nrf2 from Keap1 thereby rescuing Nrf2 from proteasomal degradation and allowing for entry into the nucleus. Additionally, involving secondary sensor proteins and the activation of protein kinase signaling pathways (MAPK-, JNK-, PKC- and PI3K-pathway), it

results in phosphorylation of Nrf2 which enhances stability and/or release from Keap1 (W.O. Osburn et al., 2008). Nrf2 translocates to the nucleus, and associates with small Maf proteins (MARE-binding components; H. Motohashi et al., 2004). After recruiting transcription co-activators to help remodel chromatin structures and facilitate formation of basal transcription machinery (Z. Sun et al., 2009; K. Ohta et al., 2007; H. Motohashi et al., 2004), the heterodimers bind to antioxidant response element (ARE) and activate the transcription of phase II enzymes like NQO1, GST, GCS (X.L. Tan et al., 2009; C. Köhle et al., 2006; see fig. 1.8.).

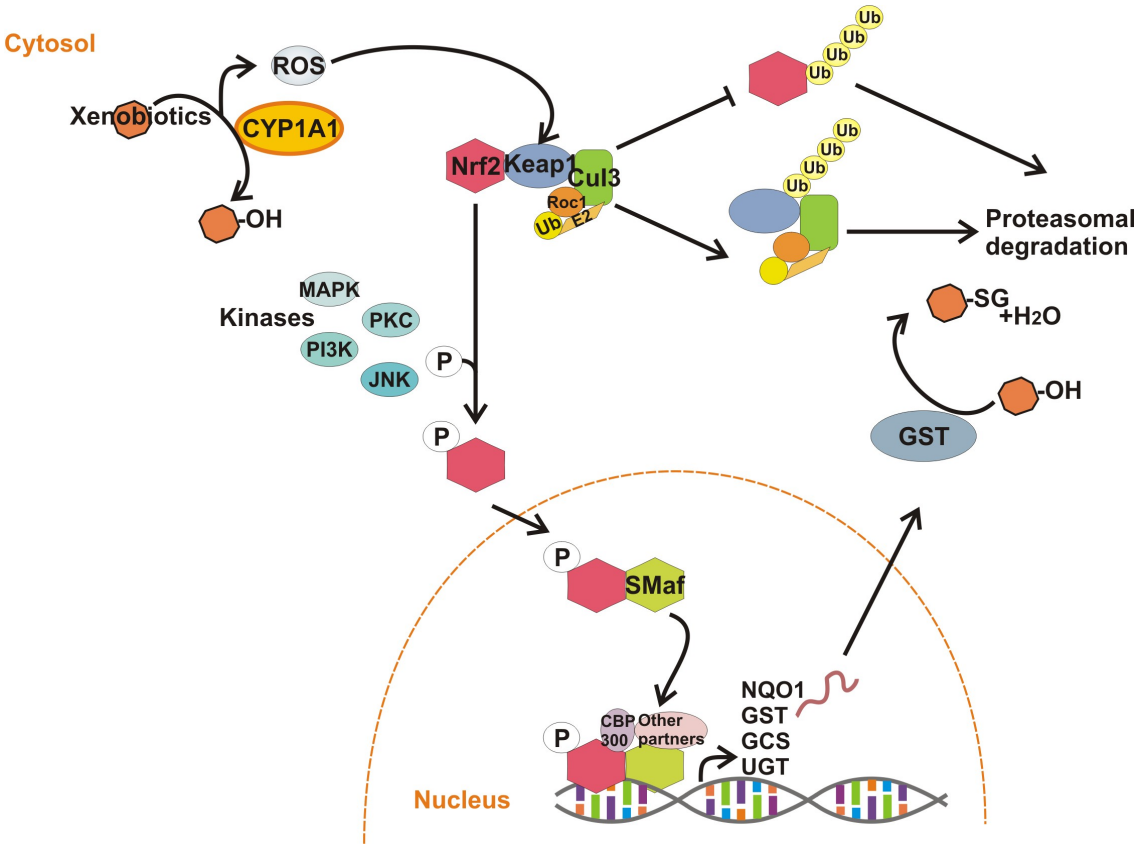


Fig. 1.8. The Nrf2 signaling pathway. Keap1 is a key regulator of the Nrf2-signaling pathway and serves as a molecular switch to turn on and off the Nrf2-mediated antioxidant response. Under basal conditions, Keap1, functioning as an E3 ubiquitin ligase, constantly targets Nrf2 for ubiquitination and degradation. As a consequence, there are minimal levels of Nrf2. Oxidative stress like ROS or chemopreventive compounds inhibit activity of the Keap1–Cul3–Roc1 E3 ubiquitin ligase. Nrf2 dissociates of from Keap1 and translocates into the nucleus. Additionally, the activation and stabilisation of Nrf2 involves phosphorylation by multiple cellular kinase pathways like that of MAPK, PKC, PI3K and JNK. Finally, Nrf2 heterodimerise with small Maf and other transcription factors and bind as a complex to the ARE, leading to enhanced expression of phase II genes (modified after A. Lau et al., 2008; W.O. Osburn et al., 2008; X.L. Tan et al., 2009).

Since the expression of GST and NQO1 enzymes is decreased in Nrf2-deficient mice (N0), they are highly susceptible to B[a]P- induced forestomach tumorigenesis (M. Ramos-Gomez et al. 2001). In a follow-up study a clearly increased level of BaP-DNA adducts in the forestomach mucosa in N0 mice compared to that of wildtyp mice was observed. These increased levels of BaP adducts were positively correlated with tumor burden (M. Ramos-Gomez et al. 2003). These results indicate that an efficient coupling of Phase I and II is very important to attenuate e.g. the CYP-mediated generation of ROS of and electrophilic metabolites and to reduce the cancer development.

1.6. Aims of the study

The fundamental subject of this study is to determine the modulatory influence of phytochemicals on the PAH detoxification by cooperative regulation signaling pathways in duodenal and colon derived cell lines. Since the major site of absorption of potential pro-carcinogens is localised in the small intestine or rather in the duodenum (90% of the total intake), the metabolism of dietary substances or contaminants in duodenal epithelial cells were in our focus. It must be pointed out that cancer of the small intestine is relatively rare compared to other gastrointestinal malignancies such as gastric cancer (stomach cancer) and colorectal cancer.

B[a]P which is a well known PAH, is found in broiled meat and smokes food and contributes to colon cancer. The adaptive function of the AhR in detoxification of xenobiotics also plays an important role in the bioactivation of pro-carcinogens like B[a]P, in particular by induction of CYP1 enzymes. Because of this an efficient and rapid elimination of potent substances depends on the connection of phase I and II enzymes. W. Miao et al. (2005) demonstrated that the Nrf2-ARE and AhR-XRE pathways directly cross-talk. XREs were identified in the regulatory region of the Nrf2. These biotransformation systems greatly facilitates detoxification and protection against oxidative/electrophile stress. This coupling could be the key mechanistic to avoid degenerative diseases and support chemo-protection of cancer (C. Köhle et al., 2006). Furthermore, other regulatory members of these pathways like the AhR repressor (AhRR) or the AhR interacting protein (AiP) could also play an important role in the controlling and stimulation of the effectively elimination of xenobiotics in the small intestine. In addition, the World Cancer Research Fund report (1997) declared that there is enough evidence to support an inverse association between dietary fruit and vegetable intake

and several cancers including colorectal cancer. Also in recent epidemiologic studies it could be shown that flavonoid intake is associated with reduced risk of colorectal cancer (E. Theodoratou et al., 2007).

Since the phytochemicals quercetin and kaempferol are mainly present in leafy vegetables, apples, onions and berries and they are the most abundant flavonoids in foods which bind to the AhR (H.P. Ciolino et al., 1999), we decided to investigate their modulatory effect on the B[a]P-induced AhR- and Nrf2-pathway in duodenum and colon derived cell lines. This modulatory effect could occur on different cellular levles like the transcription, translation or activation of proteins. Therefore, we focused on the transcriptional, protein and enzymatic activity level by means of TaqMan PCR, Westernblots and EROD assay. Since we used duodenal and colon derived cell lines, we were able to distinguish possible tissue dependent differences which are suggestive of the reason of the high colorectal risk. So far, there are no investigations available in duodenal cell lines which examined possible regulatory pathway-differences.

2. Experimental

2.2. Chemicals

Acrylamide (40%)	Amresco, Solon, Ohio, USA
Agarose	Biozym, Oldendorf, Germany
Ammoniumperoxodisulfate (APS)	Sigma, Steinheim, Germany
Benzo[a]pyrene (B[a]P)	Sigma, Steinheim, Germany
Bromophenol blue	Sigma, Steinheim, Germany
Chloroform	Merck, Darmstadt, Germany
DEPC	Armin Baack, Schwerin, Germany
Dicoumarol	Sigma, Steinheim, Germany
D-Glucose	Invitrogen, Paisley, UK
Di-methyl sulfoxid (DMSO)	Sigma, Steinheim, Germany
Ethanol	Merck, Darmstadt, Germany
Ethoxyresorufin	Sigma, Steinheim, Germany
Ethylene diamine tetra-acetic acid (EDTA)	Merck, Darmstadt, Germany
Glycerol	Sigma, Steinheim, Germany
Glycine	AppliChem, Darmstadt, Germany
Hydrogen peroxide (30%)	Sigma, Steinheim, Germany
Isopropanol	Merck, Darmstadt, Germany
Kaempferol	Fluka, Buchs, Switzerland
2-Mercaptoethanol	Sigma, Steinheim, Germany
MgCl2 Solution (PCR reagent)	Sigma, Steinheim, Germany
Quercetin-dihydrate	Fluka, Buchs, Switzerland
Sodium dodecyl sulfate (SDS)	Sigma, Steinheim, Germany
Tetramethylethylenediamine (TEMED)	Riedel-de Haën, Seelze, Germany
tert.-Butylhydrochinon	Fluka, Buchs, Switzerland
Tris-hydroxymethyl-aminomethane (Tris)	Merck, Darmstadt, Germany
Tween 20	Sigma, Steinheim, Germany

Other standard laboratory chemicals were purchased in p.a. quality from Sigma-Aldrich, (Taufkirchen, Germany), Merck (Darmstadt, Germany) and Riedel-de Haën (Seelze, Germany).

Chemicals for molecular biology

dNTPs	Stratagene, La Jolla, CA, USA
Random-primer	New England Biolabs, Beverly, MA, USA

Buffers and solutions

Phosphate buffered saline (PBS)	Invitrogen, Paisley, UK
QIAzol Lysis Reagent	Quiagen, Hilden, the Netherlands
Neutral red	Biochrom, Berlin, Germany
T-PER Tissue Protein Extraction Reagent	Pierce, Rockford, USA
Amersham ECL Advance Western Blotting Detection Reagent ECL RPN2209	GE Healthcare, Munich, Germany

Antibodies, enzymes and other proteins

Alpha-tubulin antibody	Cell signaling, Boston, USA
Antibody CYP1A1 (1A-03)	Santa Cruz, Heidelberg, Germany
AHRR polyclonal antibody (A01)	Abcam, Cambridge, USA
Goat-anti-mouse IgG, peroxidase-conjugated	Santa Cruz, Heidelberg, Germany
Goat-anti-rabbit IgG, peroxidase-conjugated	Cell signaling, Boston, USA
Milk powder	Roth, Karlsruhe, Germany
MMLV Reverse Transcriptase (incl. buffer)	Stratagene, La Jolla, CA, USA
Pre-stained-protein-ladder page ruler MBI	Fermentas, St. Leon-Roth, Germany
MagicMark™ XP Standard	Invitrogen, Paisley, UK
Spectra Multicolor broad range protein ladder	Fermentas, St. Leon-Roth, Germany

Kits

BC Assay: Protein Quantitation Kit	Interchem, Montluçon Cedex, France

Consumables

Protan-nitrocellulose-membrane	Schleicher and Schuell, Dassel, Germany
Cell culture flask	Nunc, Roskilde, Denmark
96 well culture clear plates	Costar (VWR International, Bruchsal, Germany)
96 well culture clear bottom black plates	Costar (VWR International, Bruchsal, Germany)
Cryo tubes	Roth, Karlsruhe, Germany

Reagent and centrifuge tubes, pipettes, tips,	
scrapers and filter (0,2 µm)	Sarstedt, Nürnbrecht, Germany
Micropistil	Armin Baack, Schwerin, Germany

Cell lines

HuTu-80	Cell line service, Eppelheim, Germany
IEC-6	DSMZ, Braunschweig, Germany
CaCo-2	Cell line service, Eppelheim, Germany

Cell culture medium

IEC-6:

45% Dulbecco's MEM (4.5 g/l glucose) and 45% RPMI 1640 + 10% FCS + 0.1 U/ml insulin + 1% penicillin-streptomycin-solution

HuTu-80:

DMEM: Ham's F12 medium supplemented with 2 mM L-glutamine and 10% FCS + 1% penicillin-streptomycin-solution

CaCo-2:

DMEM (high glucose) +10% FCS +1% L-Glutamine + 1% non-essential amino acids (NEAA) +1% penicillin-streptomycin-solution

All cell culture media were purchased in sterile conditions from Invitrogen.

Solution for cell culture

Fetal bovine serum	Biochrom, Berlin, Germany
Insulin solution from bovine pancreas	Sigma-Aldrich, Steinheim, Germany
L-glutamine 200mM (100x)	Invitrogen, Paisley, UK
MEM amino acids solution (50x)	Invitrogen, Paisley, UK
Penicillin-streptomycin-solution	Invitrogen, Paisley, UK
Trypsin-EDTA (1x)	Invitrogen, Paisley, UK

TaqMan® Real time PCR

All solutions, probes and consumables for the TaqMan® real time PCR were purchased from Applied Biosystems, Foster City, CA 94404, USA.

TaqMan® Real time PCR solutions

TaqMan® Universal PCR Master Mix, No UNG	4364343

TaqMan® Real time PCR probes

Housekeeping target genes	
GAPDH, human	4352934E
GAPDH, rat	4352338E
Human target genes	
AhR	Hs00169233_m1
AhRR	Hs00324967_m1
ARNT	Hs00231048_m1
CYP 1A1	Hs00153120_m1
CYP 1A2	Hs00167927_m1
CYP 1B1	Hs00164383_m1
NFE2L2 /Nrf2	Hs00975960_m1
GCS	Hs00892604_m1
AiP	Hs00610222_m1
Rat target genes	
AhR	Rn00565750_m1
AhRR	Rn01537441_m1
ARNT	Rn00562847_m1
CYP 1A1	Rn00487218_m1
CYP 1A2	Rn00561082_m1
CYP 1B1	Rn00564055_m1
NFE2L2/ Nrf2	Rn00477784_m1
GCLC (GCS)	Rn00563101_m1
AiP	Rn00597273_m1

Instruments

Blotting-chamber TE Series Biotech	Hoefer Pharmacia Biotech, San Francisco, USA
SE 260 Mini-Vertical electrophoresis-unit	GE Healthcare, Munich, Germany
Thermocycler	Biometra, Göttingen, Germany

Nanodrop 1000 Spectrophotometer	Nano Drop technologies, Wilmington, Delaware, USA
7500 Real Time PCR System	Applied Biosystems, Darmstadt, Germany
ChemiLux imager 1400	Intas Science Imaging Instruments GmbH, Göttingen, Germany
Microtiter shaker MTS2	IKA, Staufen, Germany
KMO 2 basic IKAMAG®	IKA, Staufen, Germany
Minishaker MS 2	IKA, Wilmington, USA
SpectrafluorPlus	Tecan, Crailsheim, Germany
Heraeus Biofuge fresco	Kendro, Hanau, Germany
Genius microbalance	Sartorius AG, Göttingen, Germany
Megafuge 1.0R	Kendro, Hanau, Germany
Thermo Bench HeraSafe	Kendro, Hanau, Germany
Tumbling Tables WT17	Biometra, Göttingen, Germany

Software

WinCam 2.1	Cybertech, Berlin, Germany
GraphPad Prism 5	GraphPad Software, Inc., San Diego, CA 92130 USA

Service provider

Microarray analysis	Laboratory for Genomics and Immunoregulation Program Unit Molecular Immune & Cell Biology LIMES (Life and Medical Sciences Bonn), University of Bonn

2.2. Methods

Cell culture

IEC-6 cells (rat normal small intestine; ACC 111) were grown in 45 % Dulbecco's Modified Eagle Medium (D-MEM) (4.5 g/l glucose) and 45 % RPMI 1640 containing 10 % FCS, 0.1 U/ml insulin and 1 % penicillin/streptomycin.

HuTu-80 cells (human duodenal adenocarcinoma; KC-2/HuTu-80) were grown in D-MEM/F-12 (1:1) with L-glutamine, 15 mM HEPES containing 10 % FCS and 1 % penicillin/streptomycin.

CaCo-2 cells (human colorectal adenocarcinoma) were grown in D-MEM (high glucose: 4.5 g/l) containing 10 % FCS, 1 % L-glutamine, 1 % non-essential amino acids (NEAA) and 1 % penicillin/streptomycin.

All cell lines were cultivated at 37 °C in an atmosphere with 5 % CO_2 and the adherent, epithelial cells grown as monolayer. To split the confluent monolayer, the growth-medium was removed and the cells washed with a solution containing 1 ml 0.05 % trypsin and 0.2 g/l EDTA. The trypsin/EDTA solution was removed and 5 ml of fresh trypsin/EDTA solution were added anew. Then the cultures were incubated at 37°C (for up to 10 minutes) until the cells detached from the flask. The digesting process was stopped by adding growth-medium containing 30 % FCS. The cells were transferred into a 15 ml tube, centrifuged at 102 x g for 5 minutes and the supernatant was aspirated. The cell pellet was washed with 5 ml fresh growth-medium and after a second centrifugation step, IEC-6 and HuTu-80 cells were seeded out at 1 $x10^6$ cells/ 75 cm^2 flask. CaCo-2 cells were seeded out at 3-4 x 10^6 cells/ 75 cm^2 flask. In order to avoid cellular senescence, all experiments were performed using cells with no more than five passages.

For preserving a pool of cells, 3 $x10^6$ cells were added in 1.5 ml freezing medium (growth-medium containing 10 % FCS and 10 % DMSO), transferred into a cryovial and placed on ice immediately. Afterwards, the cryovial was frozen during 24 hours at -20°C, subsequently for 24 hours at -70°C and finally stored in liquid nitrogen.

To resuscitate the deep frozen cells, the cryovial was placed into a 37°C water-bath until the medium was re-thawed. The cells were then diluted in 10-fold volume of warm culture medium, centrifuged at 102 x g for 5 minutes and the supernatant was removed. The cell

pellet was washed with 5 ml fresh culture medium and after a second centrifugation step; the cell pellet was re-suspended in 1 ml fresh culture medium and transferred into a culture flask.

Neutral-red cytotoxicity assay

The ability of healthy cells to accumulate neutral-red into their lysosomes was used to estimate a non-cytotoxic concentration of the substances to be tested. The neutral-red accumulation assay was performed according to the method of E. Borenfreund et al. (1988). Briefly, 7.5 x10^3 (IEC-6 or HuTu-80) or 15 x10^3 (CaCo-2) cells were seeded in each of the 96-well plate. 48 h after seeding, cells in each column were incubated with different concentrations of the tested substance for further 48 hours. After treatment, the medium was exchanged for one containing neutral-red (80 µg/ml) and the cells were incubated for further 3 hours. The medium was removed and the cells were washed three times with PBS to remove the non-incorporated excess dye. To bring the accumulated neutral red in the cells into solution, 200 µl 'de-stain solution' (50 % ethanol, 1 % acetic acid, and 49 % distilled water) were added to the cells, which were then incubated for 20 minutes while gently agitated. The absorbance of the solution in each well was measured at 540 nm with untreated cells as controls using a 96-well plate Tecan spectrophotometer.

Exposure of cells to the testing chemicals

Forty-eight hours after seeding (see above), the cells were incubated as described for further 48 hours with different concentrations of B[a]P (0.01-10 µM) or the flavonoids quercetin or kaempferol (0.5-10 µM). For the combination experiments each flavonoid (10 µM) was added 30 minutes before B[a]P (1 or 10 µM; see fig. 2.1.). Since the solvent used for preparation of the stem solutions must not affect the expression of the studied genes by itself, the final concentration of DMSO was set to 0.1 % (v/v) (fig. 2.2.).

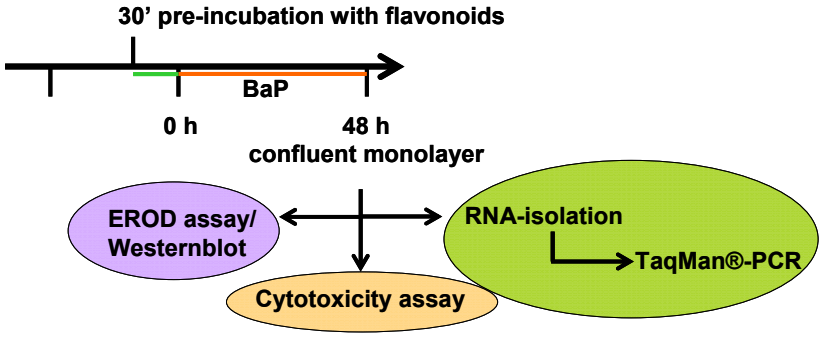


Fig. 2.1. Scheme of the experimental treatment of the cell lines and subsequent analyses.

After incubation, total RNA was isolated from the treated and untreated confluent cells by the QIAzol Lysis Reagent (phenol-chloroform method). The cell monolayer was lysed directly in the 75 cm^2 culture flask by adding 5 ml of the QIAzol. The samples were lysed with QIAzol at room temperature for at least 5 minutes. A 1 ml aliquot was thereafter vigorously mixed with 0.2 ml chloroform for 15 seconds, settled 2-3 minutes at room temperature and centrifuged for 15 minutes at 12,000 x g at 4°C. A 330 µl aliquot of the aqueous colourless upper phase was transferred to a fresh tube, mixed with 0.5 ml isopropanol, settled at room temperature for 10 min and centrifuged for 10 minutes at 12000 x g at 4°C. The supernatant was removed and the pellet was washed with 750 µl 75 % ethanol and centrifuged at 7500 x g for 5 minutes at 4°C. In order to obtain optimal RNA purity parameters a second "cleaning" step phase with ethanol was performed, the supernatant was then removed and the RNA pellet air dried and re-suspended in 20 µl DEPC water.

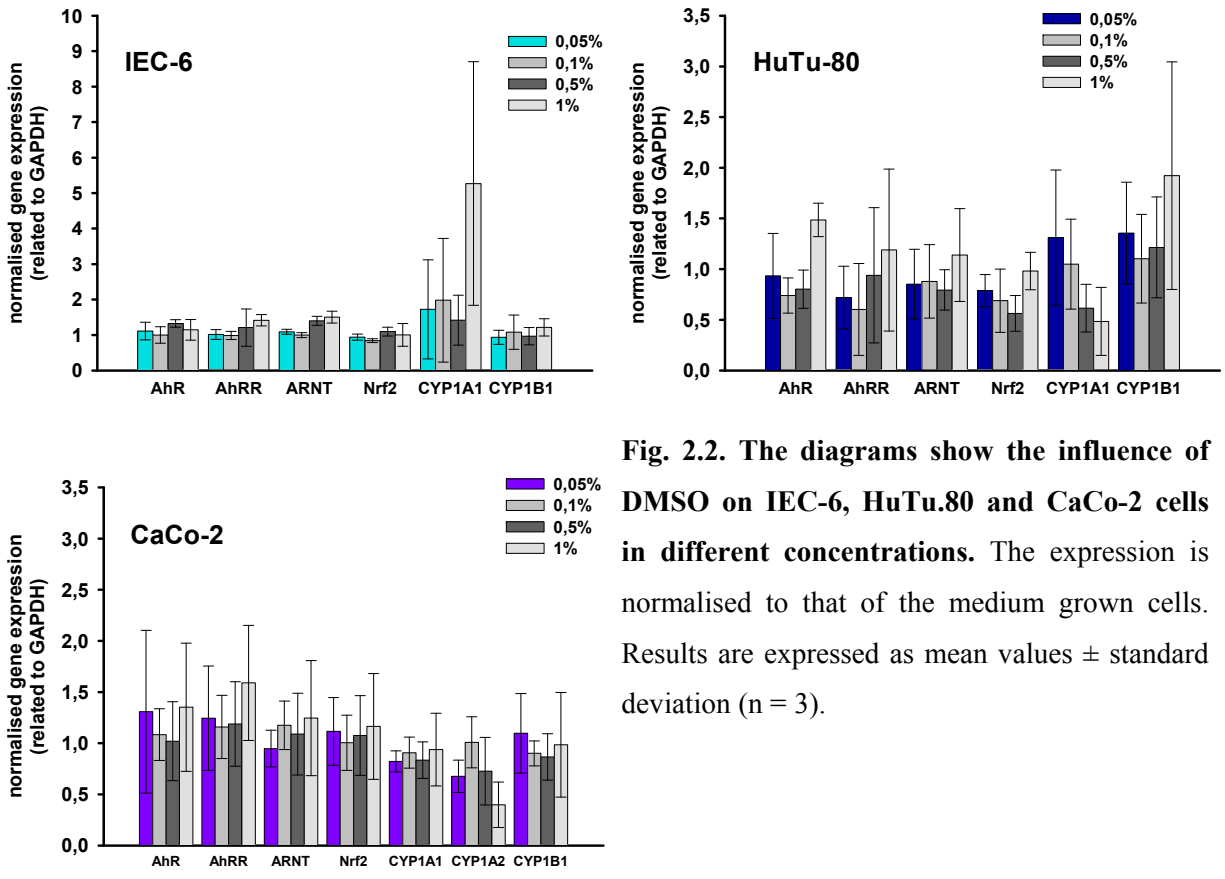


Fig. 2.2. The diagrams show the influence of DMSO on IEC-6, HuTu.80 and CaCo-2 cells in different concentrations. The expression is normalised to that of the medium grown cells. Results are expressed as mean values ± standard deviation (n = 3).

		0,05%		0,10%		0,50%		1%	
		MV	SD	MV	SD	MV	SD	MV	SD
IEC-6	AhR	1,11	0,25	1,00	0,23	1,33	0,11	1,15	0,29
	AhRR	1,02	0,14	0,99	0,12	1,21	0,53	1,42	0,16
	ARNT	1,09	0,07	1,00	0,07	1,40	0,13	1,51	0,17
	Nrf2	0,94	0,09	0,84	0,05	1,10	0,12	1,00	0,32
	CYP1A1	1,72	1,39	1,98	1,74	1,42	0,70	5,27	3,43
	CYP1B1	0,93	0,20	1,08	0,48	0,97	0,24	1,22	0,24
HuTu-80	AhR	0,93	0,42	0,74	0,17	0,80	0,19	1,49	0,17
	AhRR	0,72	0,31	0,60	0,45	0,94	0,67	1,19	0,80
	ARNT	0,85	0,34	0,88	0,36	0,79	0,20	1,14	0,46
	Nrf2	0,79	0,16	0,69	0,31	0,56	0,18	0,98	0,18
	CYP1A1	1,31	0,67	1,05	0,44	0,61	0,24	0,48	0,34
	CYP1B1	1,36	0,50	1,10	0,44	1,21	0,50	1,92	1,12
CaCo-2	AhR	1,31	0,80	1,08	0,25	1,02	0,39	1,35	0,63
	AhRR	1,24	0,51	1,16	0,31	1,19	0,41	1,59	0,56
	ARNT	0,95	0,18	1,17	0,24	1,09	0,40	1,25	0,56
	Nrf2	1,12	0,33	1,01	0,27	1,08	0,39	1,16	0,52
	CYP1A1	0,82	0,10	0,91	0,15	0,83	0,18	0,94	0,36
	CYP1A2	0,68	0,16	1,01	0,25	0,73	0,33	0,40	0,22
	CYP1B1	1,10	0,39	0,90	0,12	0,87	0,23	0,98	0,51

Table 2.1. Relative values of the detected gene expression related to the expression of GAPDH (x1000). Shown are the mean value (MV) and the standard deviation (SD).

Determination of the RNA-concentration and purity

To prevent a saturation of the reverse transcription reaction and thus to ensure reproducible results, standardised amounts of RNA were reverse transcribed. Therefore, the concentration of total RNA was measured using the Nanodrop Spectrophotometer ND 1000 according to the instructions of the manufacturer.

Only high-purity RNA (OD ratios: 260/280 = 1.9-2.0 and 260/230 = 2.0-2.2) was further used for conventional RT-PCR.

Reverse transcription

Standard reverse transcription was carried out on 5 µg high-purity DNaseI-treated RNA. For doing this, total RNA (5 µg in 19.7 µl DEPC water) was digested by 2 units of DNase-I for 10 minutes at 37°C. After the addition of EDTA (5 mM) to the sample (for protecting RNA from heat degradation), the enzyme was inactivated by heating the mixture for 10 minutes at 75°C. Then EDTA was neutralised by adding 5 mM $MgCl_2$.

For the reverse transcription reaction random priming was performed by incubating the reaction-mixture at 65°C for 5 minutes and then cooling it down to room temperature within 10 minutes. For reverse transcription, dNTPs and reverse transcriptase were added to the random priming- reaction mixture (see table below). To prevent RNA-degradation, the reaction mixture also contained 1 µl of RNase Block Ribonuclease Inhibitor. The reaction-

mixture was incubated at 37°C for one hour, and then the transcriptase was inactivated by heating the mixture to 90°C for 5 minutes.

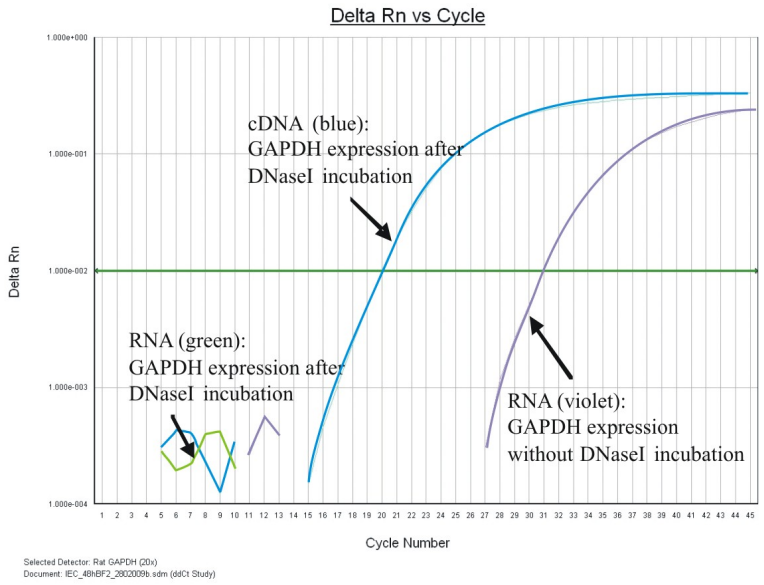


Fig. 2.3. TaqMan amplifiation curve for GAPDH of contaminated total RNA. To exclude false gene expression measurements caused by DNA contamination isolated RNA was incubated with DNaseI before starting the reverse transcription of RNA into DNA.

DNaseI -RNA mixture

19,7 µl RNA(5 µg)

1 µl DNaseI

2,3 µl DNaseI-buffer

Random priming reaction mixture

300 ng random primer (100 ng / µl)

Reverse transcriptase reaction mixture

5 µl 10x RT-buffer

2 µl 100 mM dNTPs

1 µl MMLV-reverse-transcriptase

1µl RNase blocking reagent

Human and rat TaqMan® probes for AhR, AhRR, ARNT, Nrf2, CYP1A1, CYP1A2, CYP1B1 and GADPH (as control housekeeping gene), were used for relative mRNA quantification by real-time PCR as described below.

TaqMan® Real Time PCR

The relative quantification is based on the expression levels of a target gene versus a reference gene (an untreated control). The ΔΔCt method was used to calculate the expression of the genes in relation to a housekeeping gene (GAPDH) and every experiment was replicated three times.

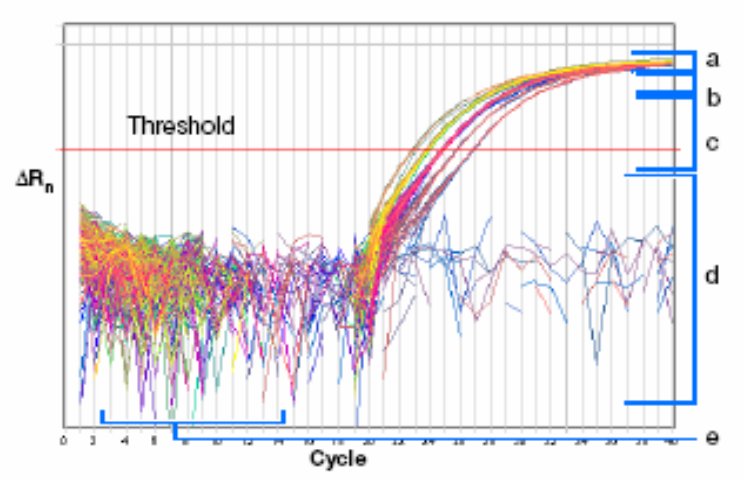


Fig. 2.4. The typical TaqMan amplification curves after 40 cycles:

- Plateau phase (a)
- Linear phase (b)
- Exponential (geometric phase) (c)
- Background (d)
- Baseline (e)

The measured amount of RNA from the gene of interest is divided by the amount of RNA from a housekeeping gene measured in the same sample to normalise a possible variation in the amount and quality of RNA among different samples.

Protein isolation

The efficiency of three methods for isolation of proteins from IEC-6 cells was evaluated. For doing this, cells were incubated 48 hours with either B[a]P (10 µM), DMSO (0.1%) or growth-medium. Then the cells were treated according to one of the following methods:

T-PER	Lysis-buffer	SDS-buffer
• Cells were scratched • admitted in 200 µl • 30 minutes incubation • centrifuged at 10,000rpm • supernatant transferred in eppendorf tube	• cells lysed in 1ml solution • cells were scratched • centrifuged at 13,000rpm • supernatant transferred in eppendorf tube	• Cells were scratched • admitted in 200 µl • suspension transferred in eppendorf tube

Lysis-buffer:

50 mM Tris-HCl, pH 7,5

0,05% Triton X-100

0,5% IGEPAL (NP 40)

150 mM NaCl

1 mM EDTA

50 mM NaF

1 mM Na_3VO_4

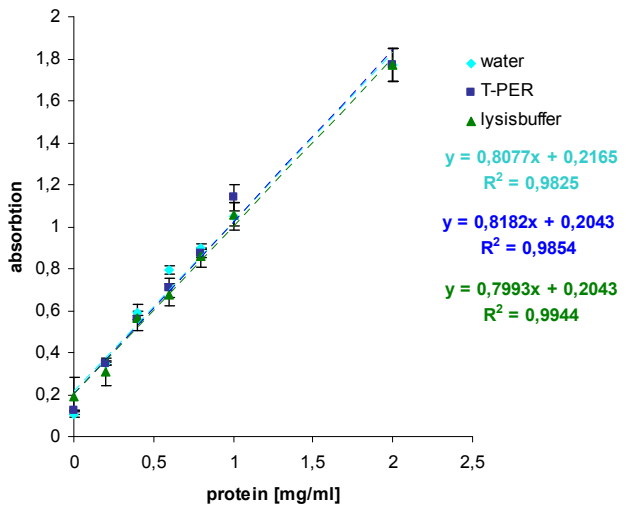


Fig. 2.5. Compatibility/interference testing of the isolation buffers, T-PER and Lysis-buffer. To exclude a buffer-mediated false determination of protein concentration we measured the absorbance (optical density; OD) of different concentrations of diluted bovine albumin dissolved in T-PER, lysis-buffer or water. Shown is the standard curve of both used buffer. Results are expressed as mean values ± S.D.

None of the tested buffers interfered with the protein concentration BC assay. Approximately, 20 µg protein of each sample were separated electrophoretically (as described below) by a SDS-PAGE (10%) and stained with the commassie-blue for 30 minutes.

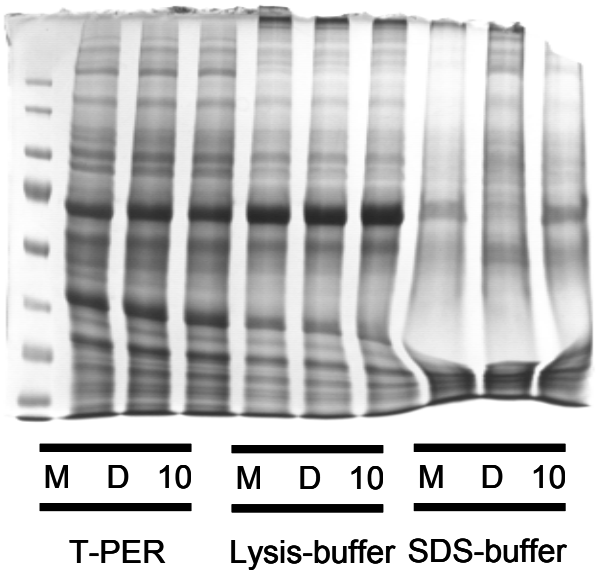


Fig. 2.6. SDS-PAGE gel stained with the commassie-blue solution shows the amount of the total protein. Untreated and treated IEC-6 cells were isolated by means of T-PER reagent, lysis- or SDS-buffer. 20 µg of the total protein were separated electrophoretically and stained with the commassie-blue. M= untreated cells (growth in medium); D= 0,1% DMSO treated cells; 10= 10 µM B[a]P treated cells.

To control the amount of isolated protein the samples were then blotted and examined for CYP1A1 and α-tubulin.

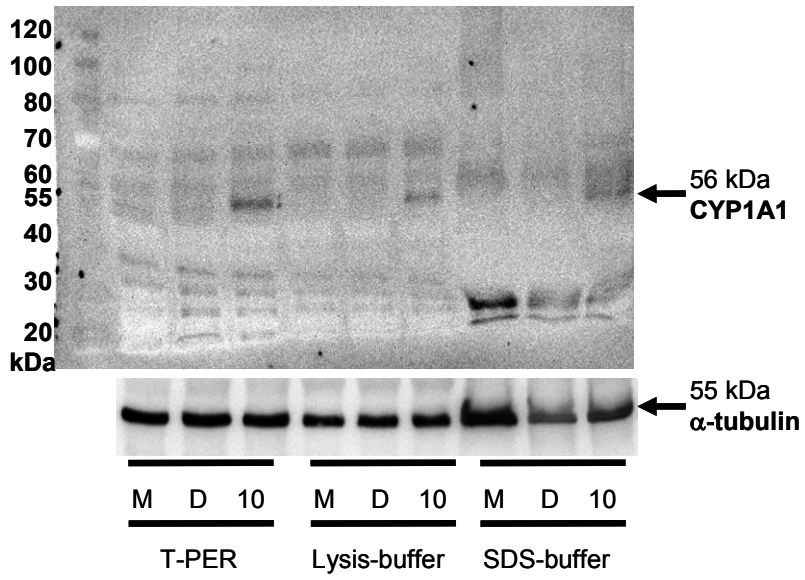


Fig. 2.7. The immuno-detection of CYP1A1 and α-tubulin. Untreated and treated IEC-6 cells were isolated by means of T-PER reagent, lysis- or SDS-buffer. 20 µg of the total protein were used of the western blot analysis. M= untreated cells (growth in medium); D= 0,1% DMSO treated cells; 10= 10 µM B[a]P treated cell s

Based on the results, T-PER solution was used for further experiment because of its good out-come efficiency and handle easiness. However, in order to enhance the protein concentration, the protocol was modified (as described below).

Total cell-lysate protein isolation

After 48 hours exposure to either B[a]P, quercetin or kaempferol (alone or in combination), the cells were trypsinised and immediately kept on ice. The detached cells were then

centrifuged at 102 x g for 5 minutes at 4°C, the supernatant was removed and the pellet was washed with 5 ml ice-cold PBS. The supernatant was removed and the cell pellet was re-suspended in 1 ml ice-cold PBS. The sample was centrifuged anew at 10,000 rpm for 10 minutes and subsequently the supernatant was removed. The cell pellet was homogenised with the appropriate amount of T-PER, settled for 30 minutes and centrifuged at 10,000 rpm for 5 minutes to precipitate the cell debris. The supernatant was finally collected and the protein content was determined by the BC assay (see below).

Determination of the total protein concentration of cell-lysates

The quantification of the total cellular CYP1A1 expression (see below) requires the application of well-defined protein-amounts in the separation-steps upstream of the immunological detection. The total cell-lysate protein concentration was determined by BC Assay (Protein Quantisation small Kit) with bovine serum albumin as standard.

A 20 µl aliquot of the total cell-lysate protein (1:10 and 1:100 dilutions), as well as of each of the protein standards were pipetted three times into a 96-microplate well. The BC Assay Working Reagent (WR) was prepared by adding 1 part of reagent B to 50 parts of reagent A. 200 µl of the WR were added to each well and the plate was incubated at 37°C for 30 minutes. After cooling the plate to RT, the absorbance (optical density; OD) was measured at 570 nm using a Tecan SpectrafluorPlus spectrophotometer.

SDS polyacrylamide gel-electrophoresis (SDS-PAGE)

As a prerequisite for immuno-detection, the total cell-lysate proteins were separated by a discontinuous, denaturing gel-electrophoresis with SDS as detergent according to U.K. Laemmli (1970).

SDS-sample-buffer:

125,5 mM Tris
0,289 mM bromophenol blue
0,138 mM SDS
40% (v / v) glycerol
10% (v / v) 2-mercaptoethanol (=5,704 mM)

Preparation of samples

The total cell-lysate protein was diluted in T-PER Tissue Protein Extraction Reagent and then 1:1 mixed with SDS sample-buffer (U.K. Laemmli, 1970) resulting in a final protein content of 50 µg for each sample. Samples were boiled for 5 minutes and afterwards kept on ice.

Standards

As a standard for the identification of CYP1A1 expression in the target cells, liver-microsomes from rats (previously exposed to 3-methylcholanthrene; see P.H. Roos et al., 2002), were used. The latter were enriched in CYP1A1, although the absolute CYP1A1-content being unknown. In the following, this preparation is referred to as "3-MC standard". On each gel, 0.01 and 0.02 µg of standard-protein were added.

Molecular weight-marker

As a molecular weight marker for the immunological protein-detection, 4 µl of the Pre-Stained-Protein-Ladder Page Ruler protein mixture and 4 µl of the MagicMark™ XP Western Protein Standard were added for each electrophoresis run.

Preparation of gels

SE 260 Mini-Vertical Unit were used to prepare discontinuous 10 x 10.5 cm gels with either 10 or 7.5 % stacking gel and 10 % separating gel, according to U.K. Laemmli (1970). The following buffers and solutions were used:

Acrylamide A:

3,5 M acrylamide

29,2 mM methylene-bis-acrylamide

Seperating-gel buffer:

750 mM Tris-HCl, pH 8,8

Acrylamide B:

3,5 M acrylamide

43,46 mM methylene-bis-acrylamide

Stacking-gel buffer:

250 mM Tris-HCl, pH 6,8

Ammonium peroxodisulfate solution (APS, freshly prepared):

0,3 M ammonium peroxodisulfate

SDS-solution:

10 % SDS in H_2O

The separating and stacking gels were prepared from the fore mentioned solutions (see table 1.2.), de-gassed by means of a water jet pump and polymerised by the addition of TEMED and APS-solution. Usually, 4 or 10 gels were poured at once and were used within 4 weeks after preparation.

	Separating gel			**stacking gel**
	10% gel	**7,5% gel**		
acrylamide A	3,5 ml	2,65 ml	**acrylamide B**	720 µl
separating gel buffer	4,5 ml	4,5 ml	**stacking-gel buffer**	1,875 ml
SDS-solution	180 µl	180 µl	**SDS-solution**	37,5 µl
destilled water	613 µl	1,5 ml	**destilled water**	1,06 µl
	to start the polymerisation:			
TEMED	19 µl		**TEMED**	7,5 µl
APS-solution	150 µl		**APS-solution**	50 µl

Gel electrophoresis

The separation of total cell-lysate proteins was performed in the SE 260 Mini-Vertical electrophoresis-unit from GE healthcare. Gels were run with a constant voltage of 160 V using the following running-buffer:

Running-buffer

25 mM Tris-HCl, pH 8,3

190 mM glycine

3,5 mM SDS

Blotting-procedure

The electrophoretically separated proteins were immediately transferred to a Protan nitrocellulose-membrane by electroblotting (1 h, 400 mA) using the wet-blotting chamber TE Series Biotech and the Tris-glycine-methanol-buffer according to H. Towbin et al. (1979).

Blotting buffer

37,5 mM Tris pH 8,3
0,2 M glycine
13,3 % (v/v) methanol

Immuno detection

After blotting, the membrane was incubated 30 minutes in blocking solution, washed three times with PBS-Tween buffer (PBST) and over-night incubated with the primary antibody for CYP1A1 (1:1000 dilution) in PBST containing 10 % milk at 4°C under gentle agitation. Then, the bolts were washed three times with PBST buffer and incubated 1 h with the goat anti-mouse IgG- horseradish peroxidase-conjugated secondary antibody (1:3000 dilution). The blots were then washed with PBST and bands were visualized using an enhanced ECL™ Western Blotting Detection Reagents (GE-Healthcare, Amersham).

To confirm equal protein loading in each well, all blots were controlled by a subsequent incubation with an α-tubulin antibody. For doing so, all blots were washed with distilled water for 5 minutes and incubated in stripping solution (0.2 M NaOH) for 5 min followed by another 5 minutes washing step with distilled water. After that, the blots were blocked in a 10 % milk PBST solution for at least 30 minutes and the blots were then incubated with the primary rabbit α-tubulin antibody (1:3000) over-night at 4°C under gentle agitation. Goat anti-rabbit IgG horseradish peroxidase-conjugated antibody was used as a secondary antibody.

PBS-Tween (PBST)

0,1 % Tween 20 (v/v) in PBS

Blocking solution

10 % (w/v) milk powder in PBS

EROD activity

The ethoxyresorufin-O-deethylase assay was performed according to A. Behrens et al. (1998) in 96-well plates with modifications. In brief, after exposure with B[a]P, quercetin, kaempferol or in combination, the medium was removed and the cells were washed twice with pre-heated to 37°C PBS. The cells were then incubated with 8 µM ethoxyresorufin and 9 µM dicoumarol in 200 µl PBS. For kinetic determinations of the enzymatic activity, the emitted fluorescence was measured at 37°C each 5 minutes during a 30 minutes period in a plate spectrometer Tecan Spectral Plus (excitation 510 nm / emission 590 nm). Afterwards, the cells were washed again twice with PBS and 20 µl 0,1% Triton-X 100 were added to the cells and frozen by -70°C for at leased 10 minutes. The enzymatic activity was related to the protein content of each well, performed by the BC assay.

Microarray analysis

Microarray analysis provides an insight into the RNA expression of 22,525 genes and offers hints of possible relevant signal pathways within the analysed cell line. Since the IEC-6 cells are rare used for detoxification analysing of substances we also send isolated RNA of untreated and treated IEC-6 cells for microarray analysis to the University of Bonn (see Service provider).

3. Results

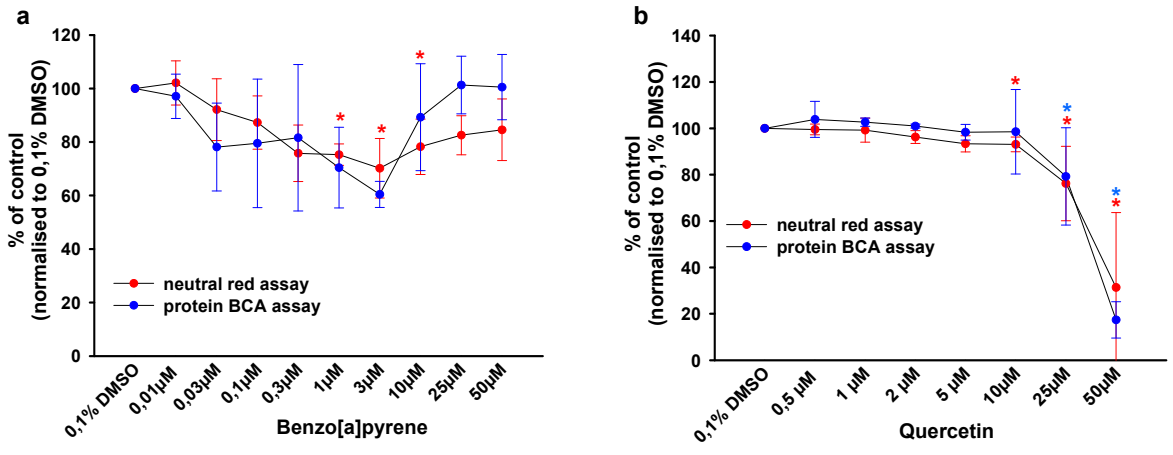
3.1. Effects of B[a]P and flavonoids on Caco-2 cells

3.1.1. Cytotoxicity of B[a]P and flavonoids on the CaCo-2 cell line

We used the neutral red (NR) and the protein content assay (BCA method) in order to determine a non cytotoxic concentration range of the carcinogen B[a]P and the flavonoids quercetin (Q) and kaempferol (K). The combination of the two methods applied to the same cells provides two distinct parameters of cell survival in the culture system. Living cells take up the neutral red dye and accumulate it within their lysosomes. Since dead cells can still be attached to the plate, the protein content and NR-stained cells are not necessarily overlapping (E. Borenfreund et al., 1988; G. Ciapetti et al., 1996; G. Fotakis et al., 2006).

Fig. 3.1. presents the dose-response curves for the cell viability and the protein content after 48 hours incubation with each of the substances (0-50 µM). B[a]P-treated cells showed a cytotoxic effect (fig. 3.1.a). Viability decreased to 70 % - 78 % ($P < 0.05$) and the protein content to 60 % - 89 % ($P > 0.05$) of the control after B[a]P treatment in the concentration range 1-10 µM. Quercetin, but not kaempferol, showed a cytotoxic effect in the neutral red assay with concentrations > 10 µM (93±3 % compared to the control; fig. 3.1.b and c). Cells exposed to 25 µM of each of the flavonols presented a significant difference in the accumulation of the dye (76.2±16 % with Q; 75±31.6 % with K) or in their protein content (79.3±21 % with Q; 81.5±35.1 % with K) compared to the control. However, CaCo-2 cells showed clear cytotoxic effects after exposure to 50 µM of any of the flavonols: viability 31.4±32.3 % and 8.8±2.2 %; protein content 17.4±7.8 % and 25.2±14.2 % of the control value, with either quercetin or kaempferol, respectively.

Effect of benzo[a]pyrene, quercetin and kaempferol on cell viability and protein content



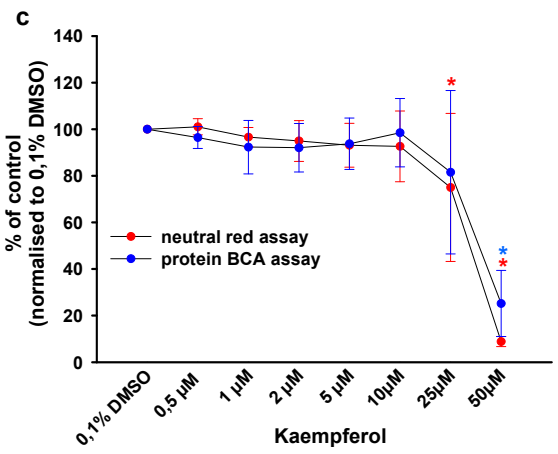


Fig. 3.1. Cytotoxicity of B[a]P and flavonoids in the CaCo-2 cell line. CaCo-2 cells were incubated with varying concentrations of benzo[a]prene (a), quercetin (b) and kaempferol (c) for 48 h, and then neutral red accumulation and BCA protein staining were measured. Results are expressed as percentage of control values ± S.D. (n = at least 3, $P < 0.05$. One sample Student's t-test). Values significantly different from the control are marked with an asterisk (red asterisks for the neutral red assay, blue asterisks for the BCA protein assay).

To characterise the modulation of the cytotoxic B[a]P-induced effect by the flavonoids in this cell line we combined 10 µM B[a]P with either 10 or 25 µM of each flavonoid. With the combination 10 µM B[a]P + 10 µM Q or K CaCo-2 cells showed a viability of 58.9±10.7 % and 56±9.2 % of the control, respectively. The protein content decreased to 80.1±33.1 % and 76.4±20.3 % of the control, respectively. The combination 10 µM B[a]P + 25 µM Q or K reduced the amount of living cells in the NR assay to 28.4±14.7 % (B[a]P + Q) and 15.1±3.1 % (B[a]P + K) compared to 0.1% DMSO. In a similar way, the protein content sunk to 38.7±15 %(B[a]P + Q) and 42.1±20.6 % (B[a]P + K) of the control (fig 3.2. a and b). In CaCo-2 cells, the combination 10 µM B[a]P + 25 µM Q or K was as toxic as the positive control (3 % H_2O_2 for 48 h). Furthermore, we obtanied a good correlation between NR assay and protein content of the treated cells before staining, providing a qualitative evaluation of the results (fig. 3.3.).

In conclusion, because of its slight cytotoxicity, we were able to use B[a]P alone in concentrations up to 50 µM for 48 hours in the Caco-2 cells. The flavonoids could be used in a concentration of up to 25 µM. For the combination experiments only 10 µM B[a]P and 10 µM of the flavonols showed a slight cytotoxic effect.

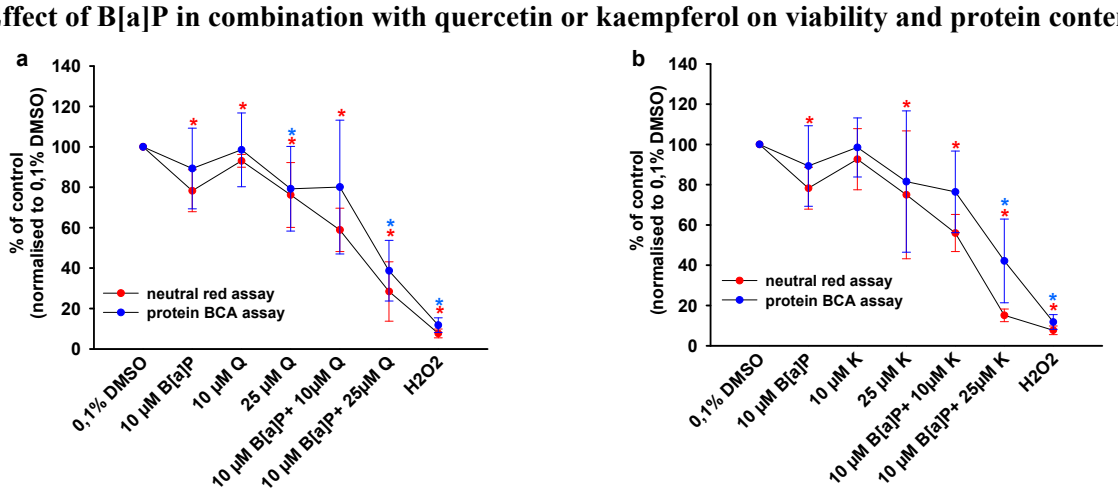


Fig. 3.2. Cytotoxicity of B[a]P in combination with quercetin or kaempferol in CaCo-2 cells. CaCo-2 cells were incubated with varying concentrations of benzo[a]prene combined with quercetin (a) or kaempferol (b) for 48 h, and then neutral red accumulation and protein content were measured. Results are expressed as percentage of control values ± S.D. ($n \geq 3$, $P < 0.05$. One sample Student's t-test). Values significantly different from the control are marked with an asterisk (red asterisks for the neutral red assay, blue asterisks for the BCA protein assay).

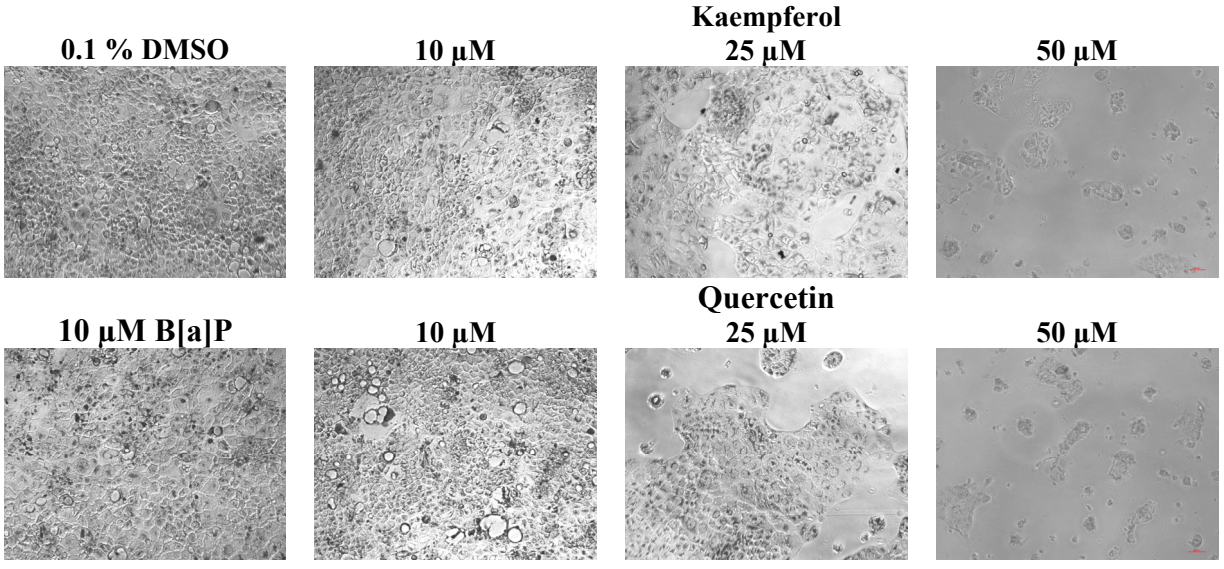


Fig. 3.3. Micrographs of treated CaCo-2 cells showing the cytotoxic effect of the tested substances. See text for details.

3.1.2. Effects of B[a]P, Q and K on gene expression in CaCo-2 cells: kinetics

The effect of B[a]P, Q and K on the gene expression of the members of the AhR- and Nrf2-pathways is clearly time-dependent. Because of this, CaCo-2 cells were incubated for 4, 24 and 48 hours with 10 µM of each of the substances. Gene expression was measured by semi-quantitative TaqMan® Real time PCR.

After 4 hours, exposure to B[a]P caused a rapid induction of almost all detected members of the fore mentioned pathways (e.g. CYPs, AhR, Nrf2, GCS and ARNT), an effect that was still observed after 48h of exposure. AhR mRNA was induced approximately 4-fold of the control level. Also, Nrf2 expression was induced up to 3-fold of the DMSO control. The transcription of the CYPs 1A1, 1A2 and 1B1 genes was strongly induced by B[a]P, being even ~70-fold of the control. The expression of the ARNT mRNA was not affected by the carcinogen, whilst the AhRR mRNA transcription seemed to be slightly induced after 4h incubation although it was reduced at later time-points (fig. 3.4.a)**.**

Quercetin induced the AhRR mRNA expression about 2 fold of the control after 48h while AhR gene transcription seemed to be slightly induced at all time-points, i.e. between 4h and 24h. Only the expression of CYP1A2 mRNA showed a clear induction by 10 µM Q (fig.3.4.b). As shown in fig 3.4.c, kaempferol induced only the transcription of CYPs. CYP1A1 and CYP1B1 were increased 6.7- and 2.2-fold, respectively, already after 4h. This effect was even stronger after longer exposure times. After 48h incubation CYP1A1 and -1B1 mRNA expression reached an induction of 81.8- and ~10-fold of the DMSO control, respectively. The transcription of the CYP1A2 gene was induced 15- and 5-fold of the control after 4 and 48h incubation.

In conclusion, both flavonols and B[a]P were able to influence the gene expression of members of the AhR- and the Nrf2-pathways which is visible already after 4h of exposure. However, the strongest effect was seen after incubation for 48 hours. Considering also the results of the cytotoxicity assays, this lead us to use an 48 hour incubation time for further experiments.

Time dependent gene expression pattern in CaCo-2 cells upon exposure to B[a]P and flavonoids

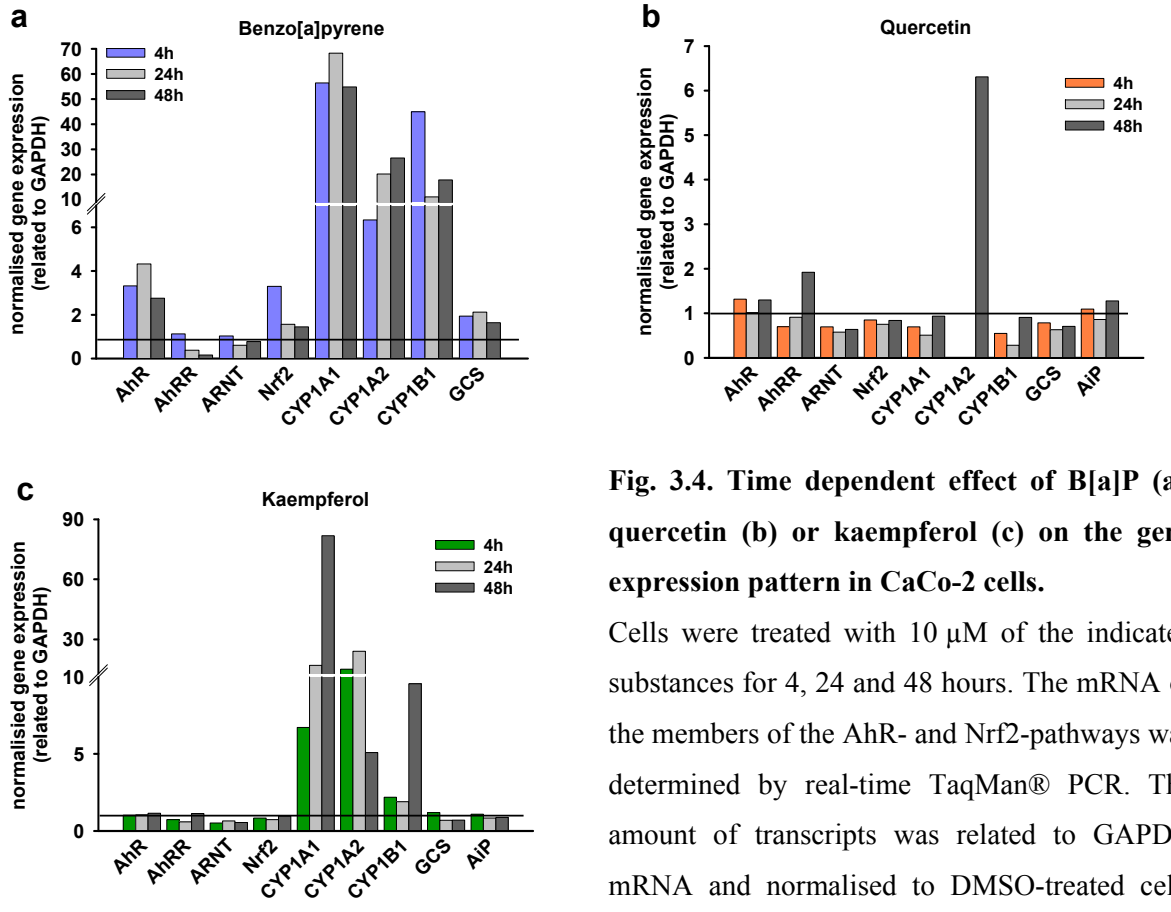


Fig. 3.4. Time dependent effect of B[a]P (a), quercetin (b) or kaempferol (c) on the gene expression pattern in CaCo-2 cells.

Cells were treated with 10 µM of the indicated substances for 4, 24 and 48 hours. The mRNA of the members of the AhR- and Nrf2-pathways was determined by real-time TaqMan® PCR. The amount of transcripts was related to GAPDH mRNA and normalised to DMSO-treated cells (DMSO =1). For the analysis the $2^{-\Delta\Delta Ct}$ method was used (n = 1).

3.1.3. Effects of B[a]P, Q and K on gene expression in CaCo-2 cells: dose response

B[a]P, quercetin and kaempferol are known ligands of the AhR; nevertheless, an effective concentration of any of these substances varies between different cell lines. Additionally, since we tried to use concentrations which are present in ordinary human foods (weekly B[a]P intake vary between 0.01 and 4.0 µg/person; K. Alexandrov et al., 1996; S. H. Lioy et al., 1988), we treated CaCo-2 cells for 48 hours with B[a]P in a concentration range from 0.01 to 10 µM (as shown in fig. 3.5.a). Furthermore, we also used experimental "high" concentrations of B[a]P (10 µM-50 µM, fig 3.5.b) to confirm the effect of the carcinogen.

From the 3 compounds studied, B[a]P had the strongest effect on the expression of the CYPs. The transcription of CYP1A1 was induced 1.6-fold of the control by a concentration as low as 0.03 µM B[a]P, whilst 3µM of the PAH showed a maximal induction effect amounting to 80-

fold of the control level. In a similar way, CYP1B1 gene transcription was induced to 1.6- and ~40-fold of the control cells (DMSO) with 0.01 and 10 µM B[a]P, respectively, while the expression of CYP1A2 increased 3.4- and 8.4-fold of the control with 1 and 10 µM of the carcinogen. Likewise, the AhR mRNA expression was induced by 3 µM B[a]P (2-fold of the control). Also, Nrf2 and GCS expression appeared to be slightly induced by concentrations up to 3 µM B[a]P. ARNT mRNA levels seemed to be slightly reduced. Interestingly, 1 µM B[a]P reduced the expression of the AhRR mRNA to 50 % of the DMSO control level. As shown in fig 3.5.b, maximal induction or suppression effects by B[a]P were achieved by a concentration of 10 µM, i.e. higher concentrations of 25 and 50µM showed no further increase or decrease, respectively.

CaCo-2 cells were incubated with 10 and 25 µM of each flavonoid in order to assess effects on expression profiles. As shown in fig 3.5.c, quercetin and kaempferol induced the expression of CYPs, too. The CYP1A1 mRNA transcription was induced by both flavonoids (334.1-fold with Q and 14.2-fold of the control level with K). CYP1B1 showed a similar effect (14.2- fold of the control level with Q and 2.1-fold with K). In this experiment, 10µM of either flavonol obviously resulted in a stronger induction of CYP1A1 and CYP1B1 in comparison to a concentration of 25 µM. Similarly, quercetin increased the level of the CYP1A2 transcript (~14-fold of the control level). In contrast, kaempferol obviously did not modulate the transcription of CYP1A2 in this experiment. The other members of the AhR and Nrf2 pathways studied here were not affected by the flavonoids.

In conclusion, B[a]P clearly affected the expression of the AhR, AhRR and CYPs in a concentration of at least 1 µM, reaching a maximum with 10 µM. The synthesis of CYP and AhR transcripts was increased, but the expression of AhRR mRNA was reduced to 50 % of the control. Higher concentrations of B[a]P did not show a stronger effect. On the other hand, the flavonoids induced clearly only the expression of CYPs. 10µM of either quercetin or kaempferol showed a stronger induction effect than 25 µM did. The reason for this result could be the stronger cytotoxic effect of the flavonoids at this concentration (fig. 3.1.b and c).

Normalised gene expression pattern induced by varying concentrations of B[a]P, quercetin and kaempferol

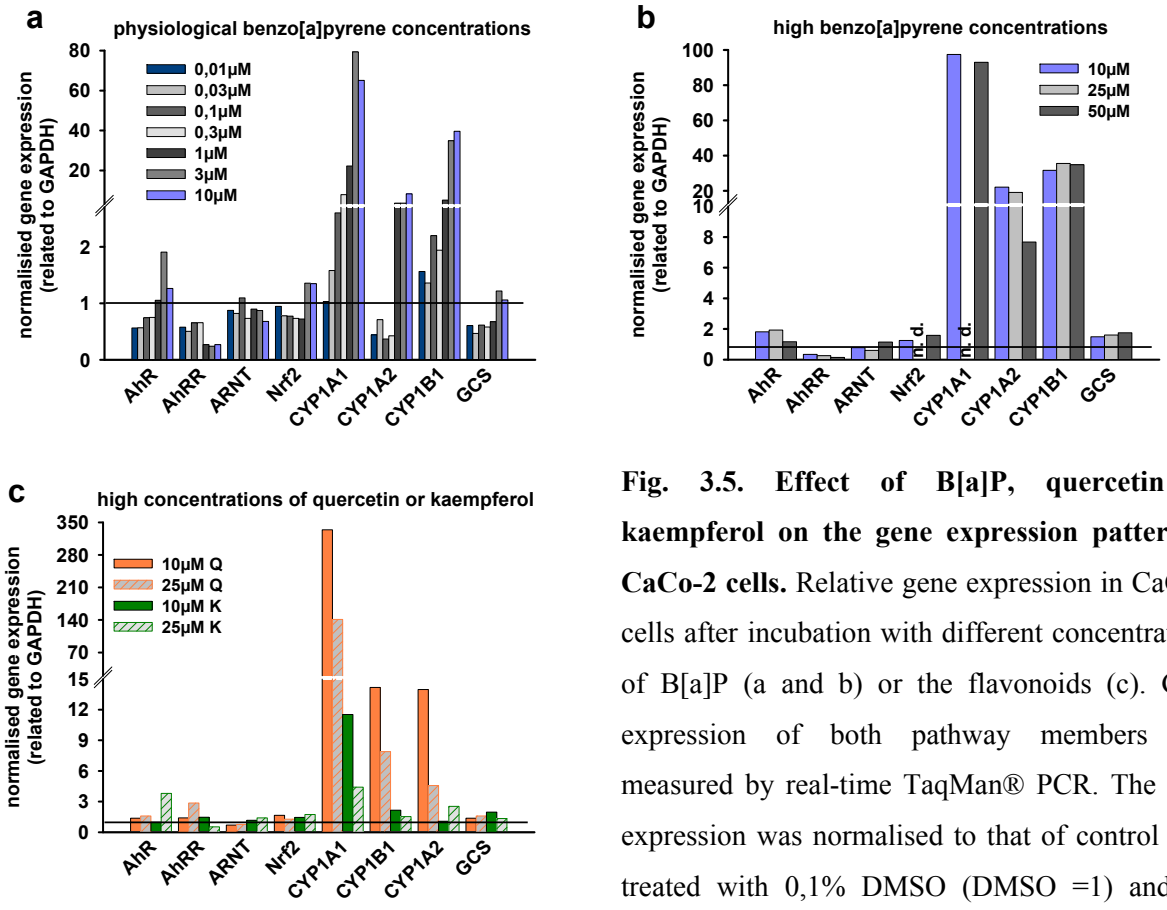


Fig. 3.5. Effect of B[a]P, quercetin or kaempferol on the gene expression pattern in CaCo-2 cells. Relative gene expression in CaCo-2 cells after incubation with different concentrations of B[a]P (a and b) or the flavonoids (c). Gene expression of both pathway members was measured by real-time TaqMan® PCR. The gene expression was normalised to that of control cells treated with 0,1% DMSO (DMSO =1) and the relative fluorescence of the transcribed genes was related to that of the house-keeping gene GAPDH. The $2^{-\Delta\Delta Ct}$ method was used for the analysis. The columns represent one experiment.

3.1.4. Influence of quercetin and kaempferol on the B[a]P-induced gene expression

For the combination experiments, we used 1 or 10µM B[a]P plus 10 µM of either flavonoid (see the results of the concentration-dependent and cytotoxicity trials). 10 µM B[a]P significantly induced the transcription of AhR (1.7-fold ±0.6 of the control). 1 µM B[a]P, quercetin or kaempferol alone did not influence the AhR mRNA expression. The combination of 1 and 10 µM B[a]P + Q did not show any increase of the AhR transcript (fig. 3.6.a). However, the combination of 1 and 10 µM B[a]P + K trended to increase the AhR expression, compared to kaempferol alone.

B[a]P lead to a decrease of the AhRR transcript level in a concentration-dependent manner (0,4-fold ±0,1 with 1 µM and 0,2-fold ±0,01 with 10 µM B[a]P), whilst quercetin induced the AhRR expression (1,5-fold ±0,3 of the control). On the contrary, kaempferol did not show any effect on the AhRR mRNA transcript. Both flavonoids, however, counteracted the suppression effect elicited by B[a]P on AhRR expression. This is also true for Kaempferol which alone did not influence AhRR transcript levels.

B[a]P led to a significant concentration-dependent reduction of the ARNT mRNA expression as well (0,7-fold ±0,04 of the DMSO control). Quercetin showed a similar effect (0,7-fold ±0,07 of the control) which was enhanced when combined with B[a]P (0,6-fold ±0,03 of the DMSO). Kaempferol by itself did not clearly change the ARNT transcript and also did not influence the B[a]P-mediated decrease in ARNT mRNA (0,8-fold ±0,1 of the control level). Both, B[a]P and the flavonoids alone or in combination did not show any noticeable effect on the AhR-interacting protein AiP at all.

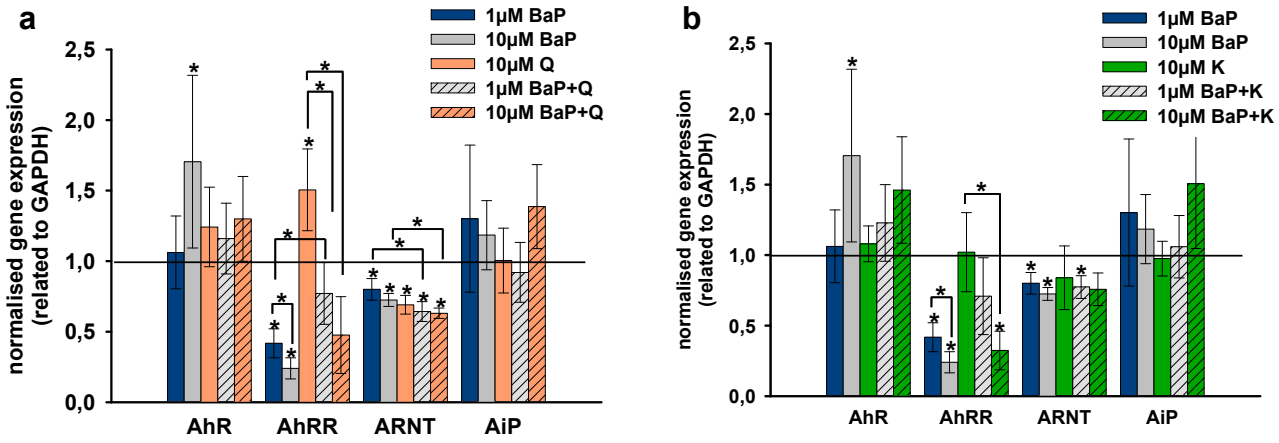
Interestingly, B[a]P and quercetin considerably increased the transcription of CYP1A1(86-fold ±26 with 10 µM B[a]P and 91-fold ±15 with Q of the DMSO control), CYP1B1 (39-fold ±19 and 11-fold ±7 of the control, respectively) and CYP1A2 (20-fold ±13 and 8-fold ±4 of the control, respectively). On the other hand, kaempferol showed no effect on the expression of CYP1A2 and CYP1B1 (fig. 3.6.e and f).

The combination 1 µM B[a]P + Q presented a tendency to additively induce CYP1A1 transcription, reaching a maximum of about 109-fold ±32.8 of the DMSO level (which, however, was not significantly higher than the expression induced by quercetin alone, see above). So, this combination had a stronger effect than 1 µM B[a]P alone on the expression of CYP1A1 (fig. 3.6.e). Even though kaempferol reduced CYP1A1 transcript, it did not counteract the induction by 10 µM B[a]P (93-fold ±19 of the control vs. 86-fold ±24 for B[a]P alone). Additionally, both flavonols slightly reduced the B[a]P-induced expression of CYP1A2 (4.5-fold ±1.4 of DMSO with B[a]P + Q and 8-fold ±5,7 of the control with B[a]P + K). Furthermore, 10 µM B[a]P + Q led to an enhancement of CYP1B1 expression but it did not reach the expression level induced by 10 µM B[a]P alone (29-fold ±12.7 of the control level). On the other hand, B[a]P + K did not show any difference in the CYP1B1 expression compared to the induction by B[a]P alone.

The second transcription factor, Nrf2 as well as its target gene GCS were induced by 10 µM B[a]P (1.4-fold ±0.1 and1.3-fold ±0.3 of the control, respectively), yet, the flavonols exhibited no clear effect on the expression of these genes. In the combination experiments, neither B[a]P + Q nor B[a]P + K showed a reduction of the B[a]P-induced Nrf2 transcription. The GCS expression presented a similar reduction tendency, but without statistical significance (Nrf2: 1.1-fold ±0.05 with B[a]P + Q and 1.1-fold ±0.04 of the control with B[a]P + K; GCS: 1-fold ±0.1 with B[a]P + Q and 1.2-fold ± 0.2 of the control with B[a]P + K, fig. 3.6.c and d).

Summarised, the carcinogen increased the expression of the CYPs in a concentration-dependent manner in CaCo-2 cells. The transcripts of GCS and the two transcription factors, AhR and Nrf2, were clearly induced by 10 µM B[a]P. However, this PAH showed a concentration-dependent suppression of AhRR and ARNT in CaCo-2 cells. Quercetin distinctly enhanced the expression of the CYPs and AhRR, but reduced the transcription of ARNT, as 10 µM B[a]P did. On the contrary, kaempferol by itself showed no effect at all. In the combination experiments we observed a mixture of modulatory effects on the gene expression. B[a]P + Q exhibited a clear antagonistic effect on the Nrf2 expression and a similar tendency was observed on the GCS transcript. An additive stimulating effect was noticed in the expression of the CYPs. However, the combination of B[a]P with quercetin showed an additive inhibition on ARNT mRNA levels and a counteracted activity on AhRR expression. Though, B[a]P + K showed only a counteracted activity on the AhRR transcript.

Influence of quercetin and kaempferol on the B[a]P-induced gene expression



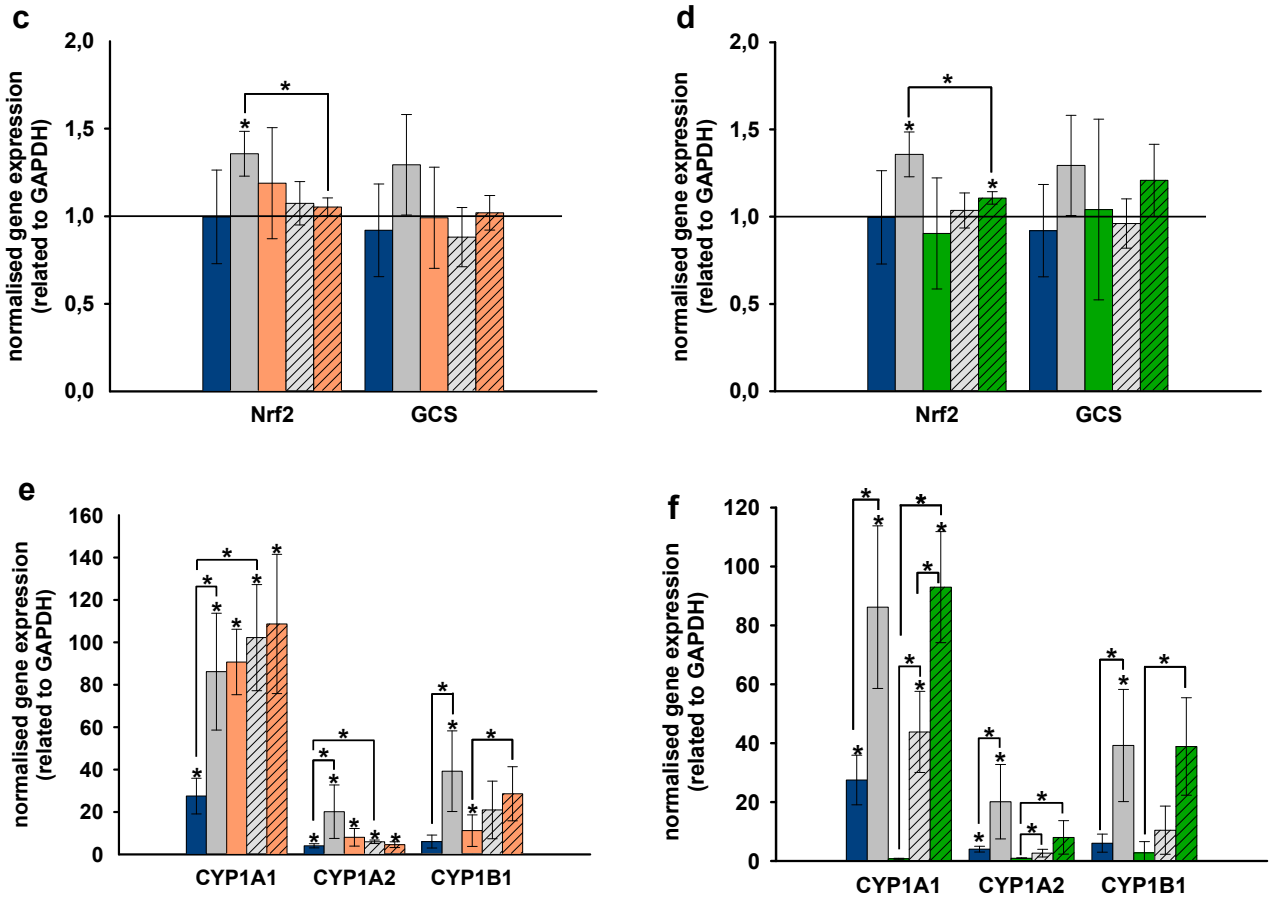


Fig. 3.6. Effect of quercetin or kaempferol on the B[a]P-induced gene expression.

Relative gene expression in CaCo-2 cells after incubation with 1 or 10µM B[a]P, 10µM of each flavonoids or a combination of B[a]P plus either flavonoids. Gene expression for members of both pathway was measured by real-time TaqMan® PCR. The gene expression was normalised to that of the 0,1% DMSO control and the relative fluorescence of the PCR products was related to that of the house-keeping gene GAPDH. The $2^{-\Delta\Delta Ct}$ method was used for the analysis. Results are expressed as mean values ± S.D. ($n \geq 3$, $P < 0.05$). Values significantly different from the control are marked with an asterisk (One sample Student's t-test). An asterisk over a column shows a significant difference compared to DMSO (DMSO = 1) whilst significant differences between the substances are shown over the bonds (Unpaired Student's t-test).

3.1.5. Influence of quercetin and kaempferol on the B[a]P-induced protein expression

B[a]P is a strong inducer of the expression of CYP1A1 protein (J. Chung et al. 2007; P.H. Roos et al. 2002; Y. Shimizu et al. 2000; M. Till et al. 1999). By western blot analysis we also showed a clear concentration-dependent induction of CYP1A1 protein by B[a]P in CaCo-2 cells after 48 hours incubation. In comparison to mRNA expression (see table 3.1.), DMSO did not induce the synthesis of CYP1A1 protein. Both 10 µM quercetin and B[a]P induced the

transcription of CYP1A1. However, the induction effect on the protein expression was not so high with Q.

CaCo-2		DMSO	1µM B[a]P	10µM B[a]P	10µM Q	10µM K	1µM B[a]P+ 10µM Q	10µM B[a]P+ 10µM Q	1µM B[a]P+ 10µM K	10µM B[a]P+ 10µM K
AhR	MV	27,74	37,34	51,57	35,86	27,58	31,57	35,05	33,25	39,63
	SD	*12,97*	*18,57*	*19,40*	*10,86*	*3,55*	*7,74*	*6,64*	*7,36*	*10,10*
AhRR	MV	0,0116	0,0049	0,0029	0,0101	0,0089	0,0050	0,0027	0,0043	0,0023
	SD	*0,0098*	*0,0026*	*0,0030*	*0,0046*	*0,0050*	*0,0026*	*0,0016*	*0,0015*	*0,0018*
ARNT	MV	3,07	2,93	2,67	1,49	2,05	1,68	1,61	1,98	1,98
	SD	*2,20*	*1,96*	*1,81*	*0,55*	*0,59*	*0,50*	*0,36*	*0,55*	*0,65*
Nrf2	MV	6,10	7,82	9,82	6,39	4,77	7,09	6,89	6,90	7,27
	SD	*3,54*	*2,28*	*5,03*	*2,23*	*0,95*	*1,95*	*1,43*	*1,85*	*1,21*
CYP1A1	MV	0,25	10,37	24,73	13,43	0,22	21,54	22,57	9,16	19,86
	SD	*0,22*	*4,97*	*9,60*	*9,05*	*0,11*	*2,65*	*1,77*	*1,83*	*4,44*
CYP1A2	MV	0,0007	0,0042	0,0134	0,0025	0,0004	0,0030	0,0023	0,0013	0,0039
	SD	*0,0009*	*0,0043*	*0,0093*	*0,0011*	*0,0001*	*0,0004*	*0,0006*	*0,0006*	*0,0026*
CYP1B1	MV	0,05	0,51	1,87	0,41	0,07	0,81	1,15	0,38	1,54
	SD	*0,04*	*0,31*	*0,88*	*0,32*	*0,09*	*0,24*	*0,24*	*0,15*	*0,40*
GCS	MV	5,18	6,11	7,72	4,36	4,05	4,75	5,39	5,17	6,51
	SD	*3,45*	*2,00*	*3,65*	*1,26*	*0,66*	*1,21*	*0,64*	*1,35*	*1,32*
AiP	MV	5,54	7,71	10,73	5,45	5,37	5,23	7,95	5,99	8,94
	SD	*0,98*	*3,20*	*7,10*	*0,55*	*0,82*	*0,75*	*0,76*	*0,39*	*2,80*

Table 3.1. Relative values of the detected gene expression related to the expression of GAPDH (x1000). Note that the values are not normalized on the levels of control cells, i.e. they do not constitute induction factors such as in the figures shown before. Shown are the mean values (MV) and the standard deviations (SD).

In the combination experiments, quercetin tends to exert an additional stimulation on induction of CYP1A1 protein by 1 µM B[a]P (fig. 3.7.a, c and table 3.2.) while there is no further stimulation at 10µM B[a]P. On the other hand, kaempferol showed no effect on the expression of the CYP1A1 protein (fig. 3.7.b and c). Nevertheless, kaempferol exerted an antagonistic effect, tending to reduce the CYP1A1 induction by 10µM B[a]P (fig. 3.7.c).

Since B[a]P reduced the transcription of AhRR and the flavonoids slightly antagonised this effect, we looked for a possible modulation of the AhRR protein expression by means of western blot analyses. As shown in fig. 3.7.a and b, the detected AhRR protein in the CaCo-2 cells was too faint to be quantified.

Summarising, B[a]P as well as quercetin clearly induced the expression of CYP1A1, while kaempferol alone had no apparent effect on the synthesis of this protein. Interestingly, unlike

kaempferol which reduced the B[a]P-induced expression of CYP1A1, the combination Q plus B[a]P showed an enhanced CYP expression. In the western blot analysis, AhRR protein was hardly detectable. We conclude that AhRR protein did not play an important role in the modulation of CYP1A1 expression by the flavonoids in CaCo-2 cells.

Influence of quercetin and kaempferol on the B[a]P-induced protein expression

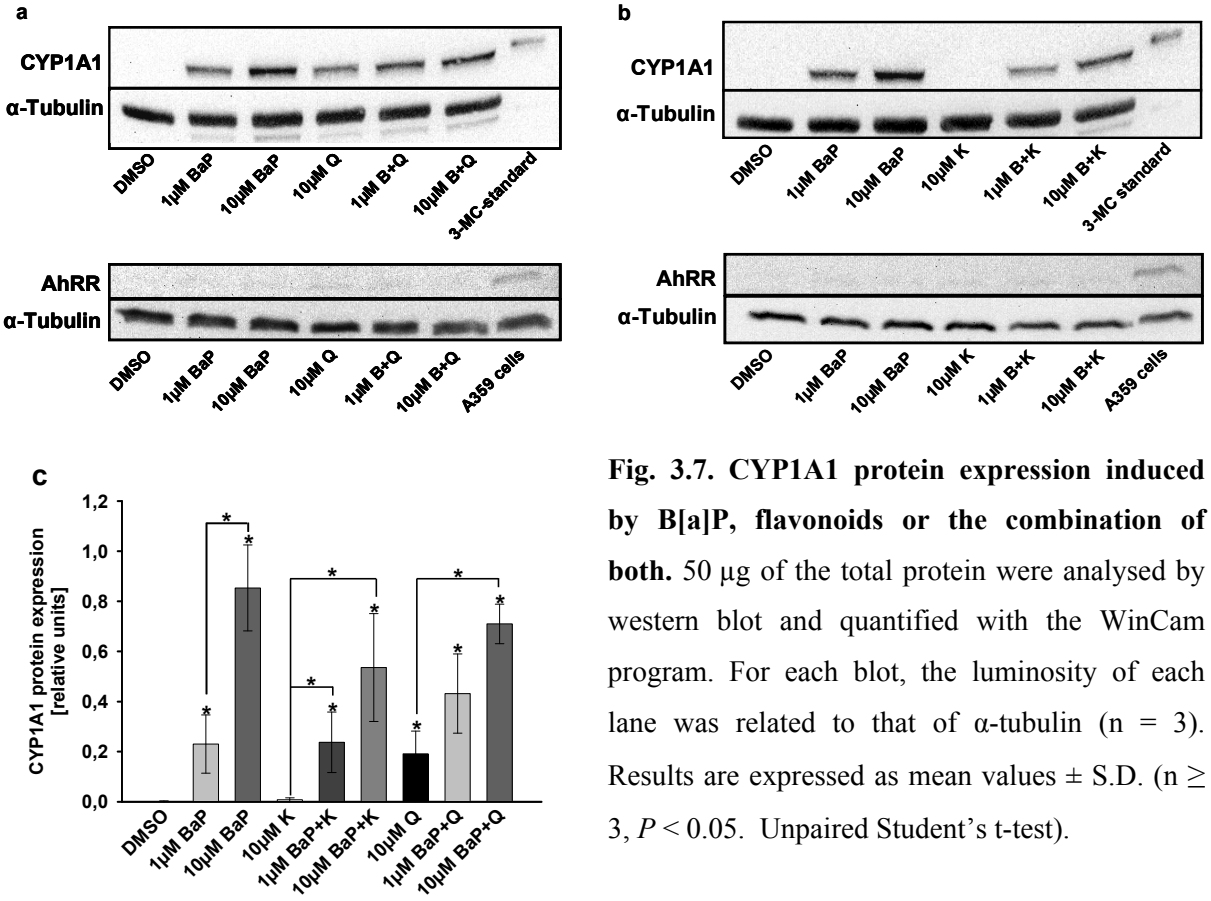


Fig. 3.7. CYP1A1 protein expression induced by B[a]P, flavonoids or the combination of both. 50 µg of the total protein were analysed by western blot and quantified with the WinCam program. For each blot, the luminosity of each lane was related to that of α-tubulin (n = 3). Results are expressed as mean values ± S.D. ($n \geq 3$, $P < 0.05$. Unpaired Student's t-test).

	DMSO	1µM BaP	10µM BaP	10µM K	1µM B[a]P +10µM K	10µM B[a]P +10µM K	10µM Q	1µM B[a]P +10µM Q	10µM B[a]P +10µM Q
Mean value	0,002	0,230	0,854	0,008	0,237	0,536	0,191	0,432	0,710
Standard deviation	0,002	0,116	0,172	0,007	0,121	0,215	0,091	0,158	0,079

Table 3.2. Relative values of CYP1A1 expression related to the content of α-tubulin.
Shown are the mean values (MV) and the standard deviations (SD).

3.1.6. Effect of B[a]P, quercetin and kaempferol on the CYP1-dependent EROD activity in CaCo-2 cells

The ethoxyresorufin-O-deethylase (EROD) assay is a common method to determine the metabolic activity of CYP1A1, 1A2 and 1B1. In accordance with the mRNA and protein expression results, DMSO- (control) and kaempferol-treated cells revealed no EROD activity (fig. 3.8.a and b). CaCo-2 cells treated with 1 µM B[a]P exhibited clear EROD activity. Also, cells treated with 10µM quercetin showed a similar induction of CYP activity (fig 3.8.a). Comparatively, 10 µM quercetin did not modify the EROD-activity of 1 µM B[a]P-exposed cells (fig 3.8.a and c). Though, 10 µM kaempferol plus 1 µM B[a]P-treated cells showed a significant reduction of the EROD activity.

Effect of B[a]P, quercetin and kaempferol on the enzymatic EROD activity in CaCo-2 cells

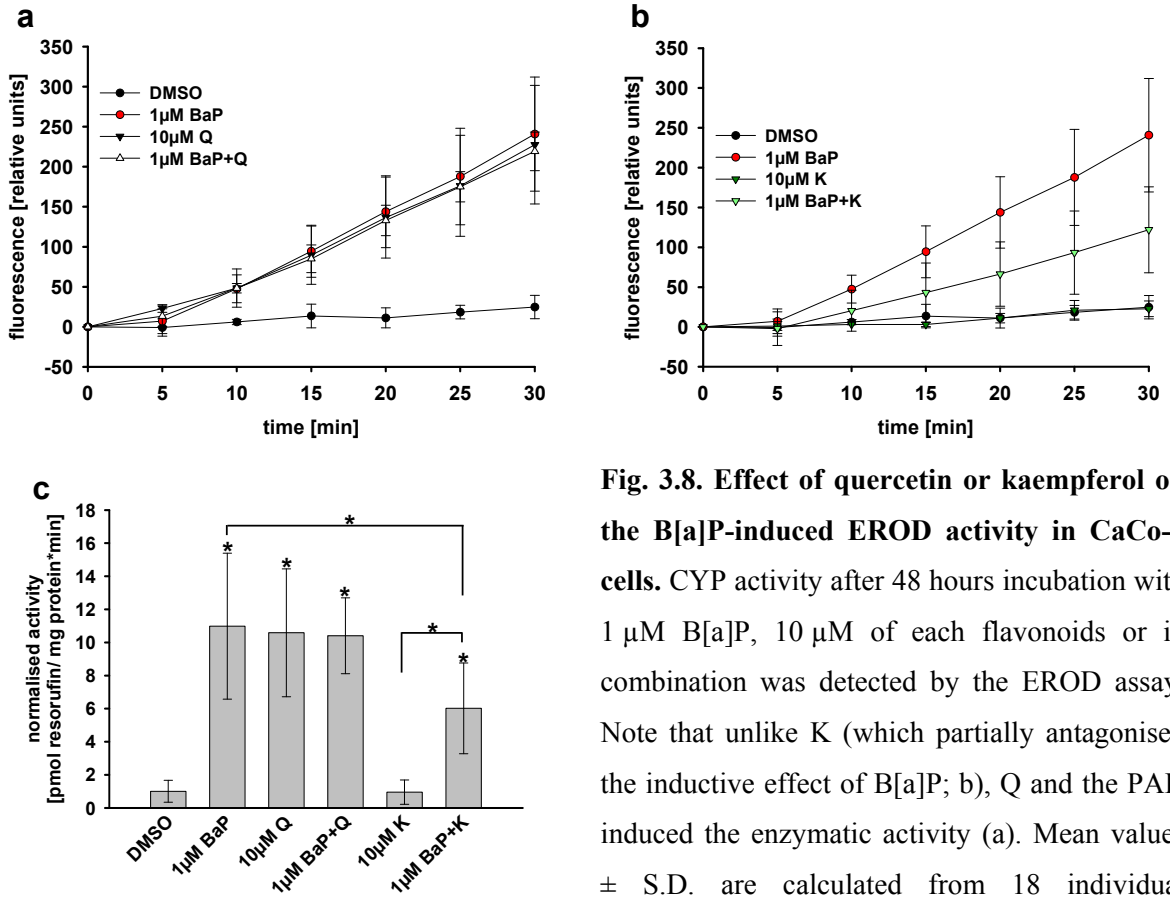


Fig. 3.8. Effect of quercetin or kaempferol on the B[a]P-induced EROD activity in CaCo-2 cells. CYP activity after 48 hours incubation with 1 µM B[a]P, 10 µM of each flavonoids or in combination was detected by the EROD assay. Note that unlike K (which partially antagonised the inductive effect of B[a]P; b), Q and the PAH induced the enzymatic activity (a). Mean values ± S.D. are calculated from 18 individual measurements. The EROD activity is normalised to the DMSO-induced activity (C). Results are expressed as means ± S.D. ($n \geq 3$, $P < 0.05$. One sample Student's t-test). Values significantly different from the control are marked with an asterisk. An asterisk over a column shows a significant difference compared to DMSO whilst significant differences between the substances are shown over the bonds (Unpaired Student's t-test).

3.1.7. Modulation of the B[a]P-induced CYP1A1 gene and protein expression by quercetin and kaempferol related to the EROD activity in CaCo-2 cells

Kaempferol and quercetin are known ligands of the AhR. Even though both flavonols have a similar structure, they showed different modulatory activity which was already described. Kaempferol did not exert any influence on the AhR- and Nrf2-pathway in the CaCo-2 cells. However, quercetin and B[a]P showed similar effects on the gene and protein expression. In figure 3.9., the clearly distinguishable differences of the modulatory activity of the flavonoids on CYP1A1 are summarised.

Modulation of the B[a]P-induced CYP1A1 gene and protein expression by quercetin and kaempferol related to the EROD activity in CaCo-2 cells

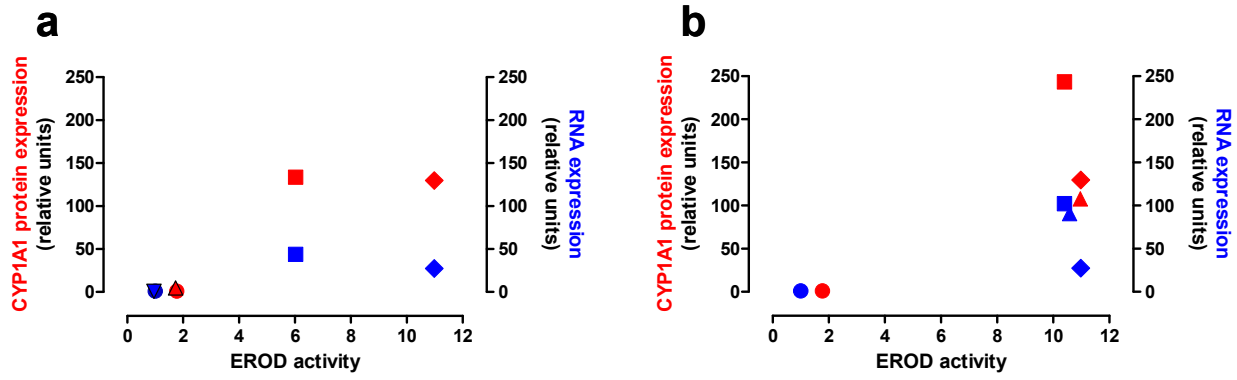


Fig. 3.9. Effect of kaempferol (a) and quercetin (b) on the CYP1A1 gene and protein expression related to the EROD activity in CaCo-2 cells. Blue Symbols represent the mRNA expression and red symbols represent the protein expression. Circle**:** 0,1 % DMSO; triangle: 10 µM kaempferol/quercetin; square: 1µM B[a]P plus K/Q; diamond: 1µM B[a]P. Unlike quercetin, kaempferol, alone or combined with B[a]P did not affect the mRNA or protein expression but reduced clearly the B[a]P-induced EROD activity of the CYPs.

Since kaempferol by itself did not induce the gene or protein expression of CYP1A1 at all, EROD activity was not detected in kaempferol treated CaCo-2 cells. B[a]P induced EROD activity as well as CYP1A1 gene expression. Kaempferol was not able to interfere in the B[a]P-induced gene or protein expression but it reduced clearly the CYP-dependent EROD activity (fig. 3.9.a). On the contrary, quercetin caused a similar increase in EROD activity compared to B[a]P treated cells; however, it caused a stronger increase of CYP1A1 transcription compared to that induced by B[a]P. Interestingly, quercetin in combination with B[a]P exhibited a similar CYP1A1 mRNA transcription compared to quercetin alone, whilst

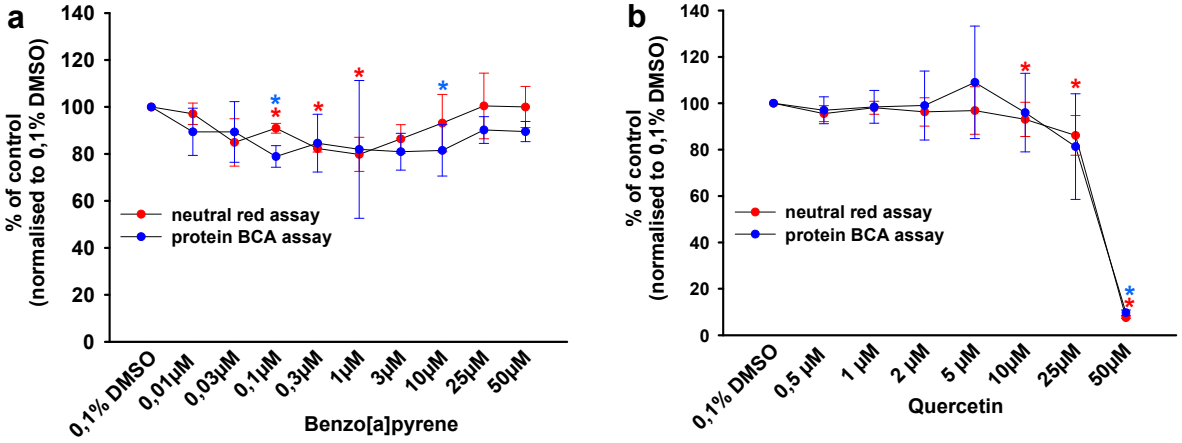
the CYP1A1 level was clearly increased without enhancement of the enzymatic activity (fig. 3.9.b).

3.2. Effects of B[a]P and flavonoids on IEC-6 cells

3.2.1. Cytotoxicity of B[a]P and flavonoids on the IEC-6 cell line

As already described for CaCo-2 cells, a non cytotoxic concentration range for B[a]P and the flavonoids was also determined for cells of the rat duodenal cell line IEC-6. As shown in figure 3.10., the dose-response curves evinced a slight but significant cytotoxic effect on the IEC-6 cells. Viability sunk from 91±2.1 % to a minimum of 79.8 ±7.3 % and protein content from 79±4.6 % to 82 ±30 % of the control after treatment with 0.1 to 1µM B[a]P, respectively. Quercetin, but not kaempferol, showed a toxic effect at 10 µM in the neutral red assay (97±7.3 % compared to the control). Cells exposed to 25 µM of any of the flavonols presented a clear cytotoxic effect visualised by the accumulation of the dye (86±8.6 % with Q; 90.2±7.7 % with K) or in their protein content (81.3±22.8 % with Q; 61.6±17.3 % with K) compared to the control. IEC-6 cells showed strong cytotoxic effects after exposure to 50 µM of any of the flavonols: with quercetin viability and protein content drop to 7.5±0.4 % and 9.8±1.1 % of the control; with kaempferol cell survival and protein content sunk to 7.7.±0.9 % and 9.2±1.9 % of the control value, respectively.

Effect of benz[a]pyrene, quercetin and kaempferol on cell viability and protein content



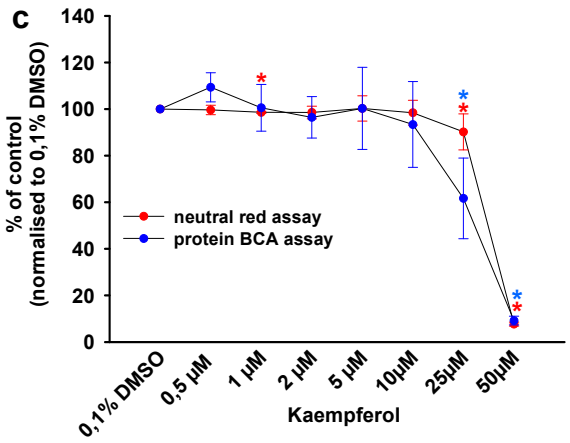


Fig. 3.10. Cytotoxicity of B[a]P and flavonoids in the IEC-6 cell line. Cells were incubated with varying concentrations of B[a]P (a), Q (b) and K (c) for 48 h. Results are expressed as percentage of control values ± S.D. (n ≥ 3, $P < 0.05$. One sample Student's t-test). Significance is marked with an asterisk (red asterisks for the neutral red assay, blue asterisks for the BCA protein assay).

With the combination 10 µM B[a]P + 10 µM Q or K, the IEC-6 cells showed a viability of 84.7±25.6 % and 85.1±7.9 % compared to the control, respectively. The protein content showed a reduction of 44.5±4.5 % and 61.7±3 % of the control, respectively. The combination of 10 µM B[a]P + 25 µM Q or K reduced the amount of living cells in the NR assay to 7±1.3 % (B[a]P+Q) and 7.6±2 % (B[a]P+K) compared to 0.1% DMSO. In a similar way, the protein content sunk to 4.4±0.4 %(B[a]P+Q) and 5.2±7.3 % (B[a]P+K) of the control (fig 3.11.a and b). As in CaCo-2 cells, the combination of 10 µM B[a]P + 25 µM Q or K was as toxic as the positive control (3 % H_2O_2 for 48 hours) in IEC-6 cells.

Effect of B[a]P in combination with quercetin or kaempferol on viability and protein content

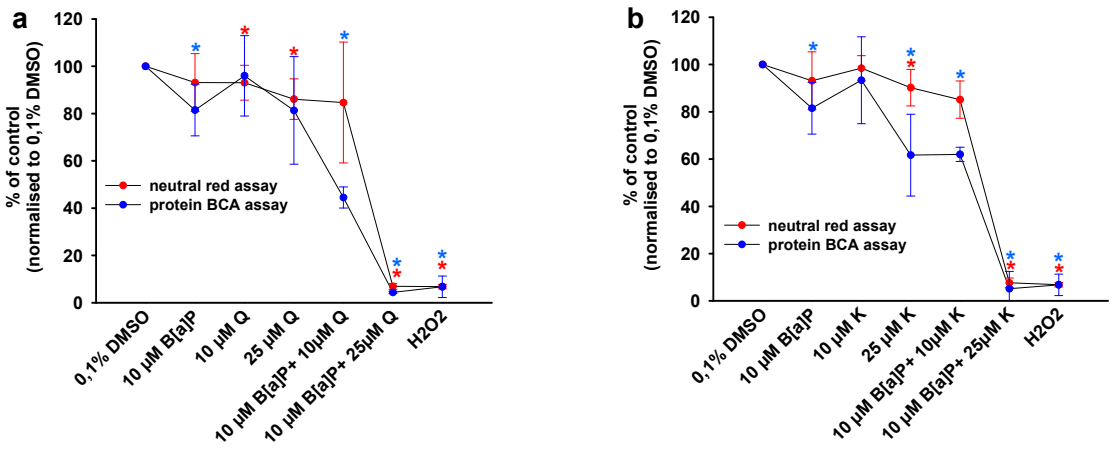


Fig. 3.11. Cytotoxicity of B[a]P in combination with quercetin or kaempferol in IEC-6 cells. IEC-6 cells were incubated with varying concentrations of benzo[a]pyrene combined with quercetin (a) or kaempferol (b) for 48 h. Afterwards, the neutral red accumulation and BCA protein staining were measured. Results are expressed as percentage of control values ± S.D. (n ≥ 3, $P < 0.05$, one sample Student's t-test). Values significantly different from the control are marked with an asterisk (red asterisks for the neutral red assay, blue asterisks for the BCA protein assay).

In conclusion, IEC-6 and CaCo-2 cells showed similar cytotoxic responses when exposed to B[a]P and the flavonoids. We were able to use B[a]P alone in a concentration up to 50 µM for 48 hours. The flavonoids alone could be used in a concentration of up to 25 µM. For the combination experiments we used 10 µM B[a]P and 10 µM of the flavonols which showed only a slight cytotoxic effect.

3.2.2. Time-dependent gene expression pattern in IEC-6 cells

We applied B[a]P, quercetin or kaempferol (10µM each) at different time points in order to detect possible previous effects on the expression of the examined genes. After 4 hours incubation B[a]P elicited a rapid induction of the expression of AhRR, CYP1A1 and CYP1B1, an effect that was still observed at the 48 hours time-point. The transcript of AhRR was increased after 4 hours incubation 3-fold ±1.8 of the DMSO control and was still observed after 48 hours (4.7-fold ±3.3 of the control). On the other hand, CYP1A1 expression exhibited a strong enhancement by B[a]P amounting to 812.4-fold ±165.5 of the control which subsequently decreased to 321.7-fold ±218.5 of the control after 48 hours. In contrast, the expression of CYP1B1 was increased after 4 hours of B[a]P-induction (3.3-fold ±1.8 of the control level) and to 7.6-fold ±1.7 of the DMSO control after 48 hours incubation. The other members of the pathways showed no clear effect of B[a]P on their expression (fig 3.12.a).

Quercetin induced different alterations in the gene expression pattern in comparison to B[a]P. As shown in fig. 3.12.b, the AhR transcript was induced 3.3-fold of the control after 48 hours. In this experiment the expression of AhRR was also strongly increased already after 4 hours (21.4-fold of the DMSO) and sunk after 48 hours of incubation to the level of the control. On the other hand, ARNT was only slightly induced after 4 hours (1.9-fold of the control) and further increased to a level of 3.3-fold of the DMSO control. Also, the expression levels of Nrf2 and CYP1B1 were increased after 4 hours (3.9-fold and 3.1-fold of the control, respectively) and 48 hours of incubation (4.9-fold and 3.9-fold of the DMSO, respectively). In contrast, CYP1A1 and AiP transcripts exhibited a strong increase after 4 hours of exposure (48-fold and 18.5-fold of the DMSO, respectively). There was no detectable CYP1A1 transcript after 48 hours incubation. The expression of AiP was still observed at this time-point but was decreased 3.4-fold of the control level. The GCS transcript level was not influenced by quercetin.

Kaempferol elicited a clear induction on the expression of AhR (4-fold of the control at the 4 hour time-point), which reached a maximum after 24 hours of treatment (5.8-fold of the DMSO level) and a minimum after 48 hours (1.6-fold of the DMSO). On the other hand, the AhRR and the AiP transcripts were increased only at the 24 hours time-point (11-fold and 4.6-fold of the control). CYP1B1 induction resembled the AhRR and AiP expression patterns. CYP1B1 was clearly induced by kaempferol after 4 and 48 hours (1.6-fold of the control on both time-points) and reached a maximum of 10-fold of the DMSO control after 24 hours of the treatment. On the other hand, the ARNT transcript showed a time-dependent induction (1.8-fold of the control at 24 hours and 3.5-fold of the DMSO-control after 48 hours the treatment). Both, Nrf2 and CYP1A1 were induced at the 4 hours time-point (5.8-fold and 4.8-fold of the control, respectively). After 24 hours of treatment Nrf2 mRNA expression was enhanced to13.7-fold of the control. The CYP1A1 transcript sunk to 3.3-fold of the DMSO level at the same time-point. After 48 hours, the expression of Nrf2 dropped to 2.8-fold of the DMSO control and CYP1A1 mRNA was not detectable in this experiment. The GCS expression did not seem to be affected by kaempferol.

Time-dependent alterations in gene expression pattern in IEC-6 cells in response to treatment

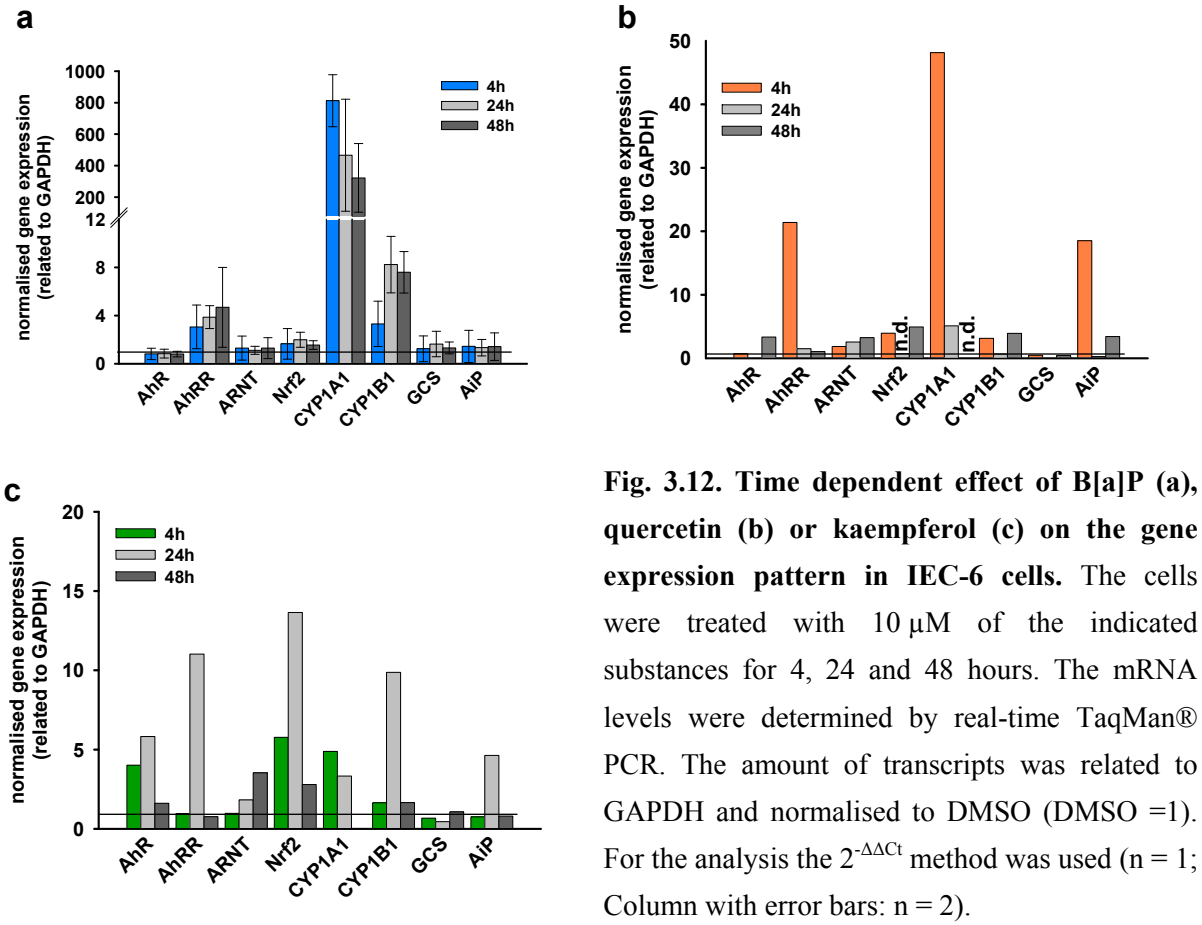


Fig. 3.12. Time dependent effect of B[a]P (a), quercetin (b) or kaempferol (c) on the gene expression pattern in IEC-6 cells. The cells were treated with 10 µM of the indicated substances for 4, 24 and 48 hours. The mRNA levels were determined by real-time TaqMan® PCR. The amount of transcripts was related to GAPDH and normalised to DMSO (DMSO =1). For the analysis the $2^{-\Delta\Delta Ct}$ method was used (n = 1; Column with error bars: n = 2).

Summarising, B[a]P clearly induced the expression of CYP1A1, CYP1B1 and AhRR. This enhanced expression was also maintained after 48 hours of exposure. The other members of the AhR pathway were unaffected by the carcinogen. Quercetin increased the transcript levels of CYP1A1, AhRR and AiP at the 4 hour time-point followed by a strong decrease in expression levels after 48 hour treatment. The expression of AhR, ARNT, CYP1B1 and Nrf2 was induced, reaching a slightly stronger level at later time-points. However, kaempferol strongly induced the expression of AhR, AhRR, Nrf2, CYP1B1 and AiP after 24 hours. The transcripts of AhR, Nrf2 and CYP1B1 were still increased at the 48 hour measurement. CYP1A1 mRNA reached its maximal expression at early time-points and sunk to no detectable levels later on. The transcription of ARNT showed a time-dependent increase by kaempferol.

Since the B[a]P-induced gene expression effect was at its highest level at the 48 hours time-point, we chose this incubation period to explore the modulatory effect of flavonoids in IEC-6 cells, too.

3.2.3. Assessing an effective concentration of B[a]P, quercetin and kaempferol for the studies with IEC-6 cells

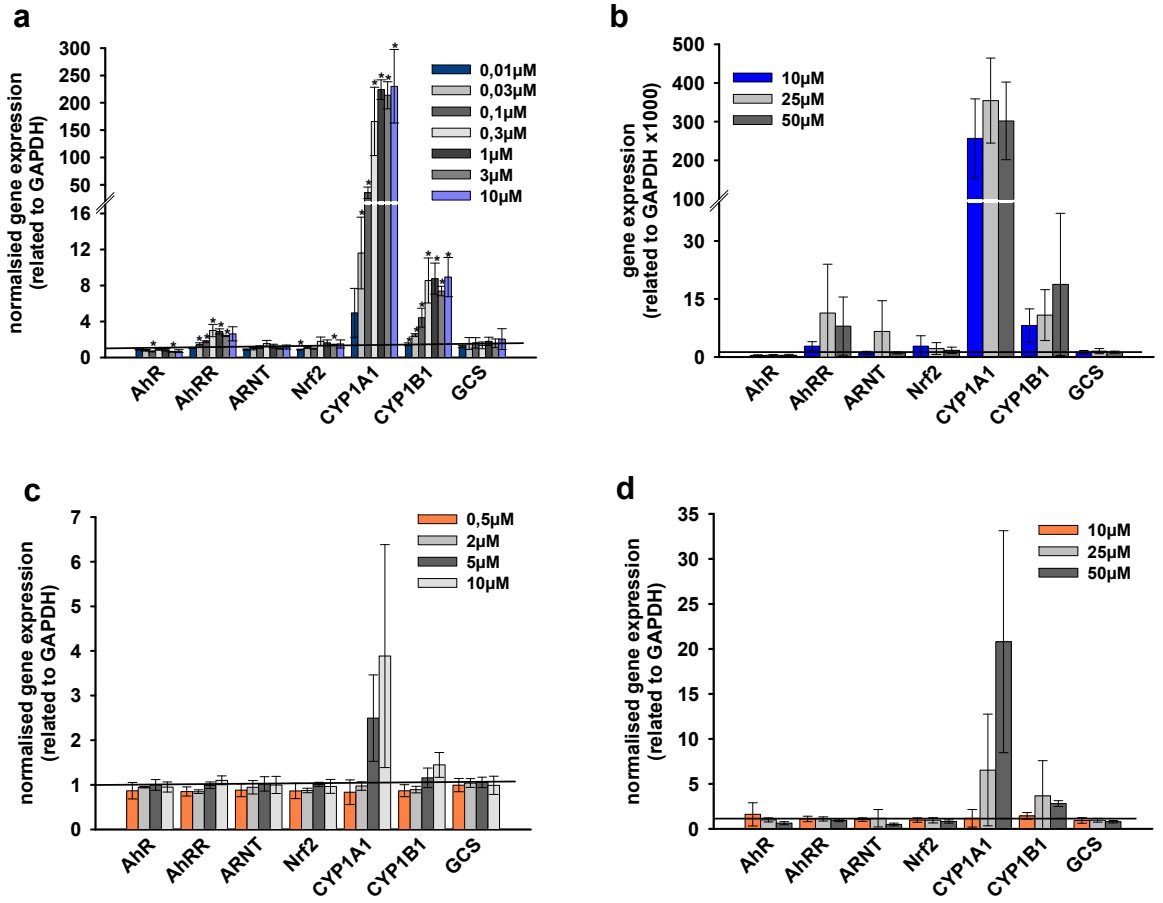
Since no data on the effects of B[a]P and flavonoids on the AhR- and Nrf2-pathways in IEC-6 cells are available, we looked for an effective concentration of each of these substances in this cell line. As already described in paragraph 3.3, we tested CaCo-2 cells with B[a]P in a concentration range from 0.01 to 10 µM as well as with experimental "high" concentrations of 10, 25 and 50 µM (see fig. 3.13.a and b). Furthermore, H.P. Ciolino et al. (1999) already showed that quercetin (0.5 µM) and kaempferol (5 µM) affected the expression of CYP1A1 in MCF-7 cells. Therefore, we incubated the IEC-6 cells with the flavonoids in a concentration range from 0.5 to 50 µM.

As shown in fig 3.13.a and b, B[a]P slightly reduced the expression of AhR (0.7-fold ±0.2 of the control with 10 µM and 0.4-fold ±0.3 of the control with 50 µM). On the other hand, the transcription of AhRR was clearly induced by 10 µM B[a]P (2.6-fold ±0.8 of the DMSO level). However, the expression of ARNT, Nrf2 and GCS did not seem to be affected by B[a]P at all. CYP1A1 and CYP1B1 are strongly induced by B[a]P even at comparatively low concentrations. Exposure to 0.01 µM B[a]P results in a 5-fold ±2.7 and 1.5-fold ±0.2 increase

compared to control cells, respectively. The induction of these two CYPs reached a maximum with 1µM B[a]P (224-fold ±18 and 8.8-fold ±1.7 of the DMSO control, respectively). The large difference in the induction factor between CYP1A1 and CYP1B1 is explained by the higher constitutive expression level of CYP1B1 compared to CYP1A1. As shown in fig. 3.13.b, additional effects of B[a]P, whether inducing or inhibitory, did not seem to be exerted by concentrations higher than 10 µM.

As already seen in the CaCo-2 cells, also quercetin induced the expression of CYP1A1 and CYP1B1 mRNA at a concentration of 10 µM in the rat cell line (3.9-fold ±2.5 and 1.4-fold ±0.3 of the control, respectively). On the other hand, other members of the AhR-pathways like AhRR were unaffected even with higher concentrations of this flavonoid (fig. 3.13.c and d). In contrast, kaempferol did not seem to affect clearly the expression of any of the studied genes in the IEC-6 cells (fig. 3.13.e and f).

Normalised gene expression pattern induced by B[a]P, quercetin and kaempferol in IEC-6 cells



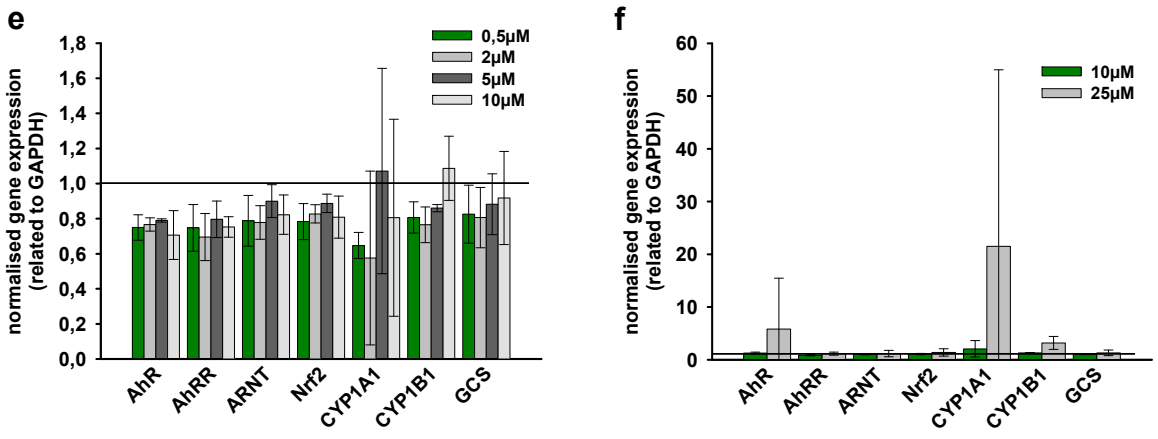


Fig. 3.13. Effect of B[a]P, quercetin or kaempferol on the gene expression pattern in IEC-6 cells. Relative gene expression in IEC-6 cells after incubation with different concentrations of B[a]P (a and b) or the flavonoids (c) for 48h. Gene expression was measured by real-time TaqMan® PCR. The gene expression was normalised to that of control cells (0,1% DMSO) and the fluorescence signals of the transcripts were related to that of the house-keeping gene GAPDH. The $2^{-\Delta\Delta Ct}$ method was used for the analysis. The columns represent at least two experiments (One sample Student's t-test).

In conclusion, B[a]P clearly affected the expression of the AhR, AhRR and the cytochromes P450 CYP1A1 and CYP1B1 in IEC-6 cells in a concentration of at least 0.01 µM, reaching a maximum at 1 µM. Higher concentrations of B[a]P did not show any further effect. The levels of CYPs and AhRR transcripts were increased, but the expression of AhR mRNA was reduced to ~50 % of the control by B[a]P. On the other hand, quercetin clearly induced the expression of CYPs only at concentrations >10µM. Since quercetin caused a cytotoxic effect (see paragraph 3.2.) we decided to utilise no higher concentration than 10 µM. Kaempferol by itself did not show any significant effect on the IEC-6 cells after 48 hours incubation although CYP1A1 expression appears slightly increased.

3.2.4. Influence of quercetin and kaempferol on the B[a]P-induced gene expression

For the combination experiments, we used 1 or 10µM B[a]P plus 10 µM of each flavonoid in IEC-6 cells, which allowed us to compare the modulatory effects of the flavonols on expression patterns in these and also in CaCo-2 cells. As shown in fig 3.14., B[a]P significantly reduced the expression of AhR in a concentration dependent fashion (0.8-fold ±0.1 and 0.7-fold ±0.2 of the control at 1µM and 10 µM, respectively). Quercetin and kaempferol did not affect the expression of the AhR mRNA. The B[a]P-induced reduction seemed to be slightly enhanced when combined with either flavonoid (0,6-fold ±0,2 of the

control with 10 µM B[a]P + Q and 0.7-fold ±0.1 of DMSO with 10 µM B[a]P + K). On the contrary, the transcript of AhRR was significantly increased by the carcinogen (3.1-fold ±0.7 with 1 µM and 3.5-fold ±1.4 with 10 µM of the DMSO level). Quercetin alone slightly but significantly induced the expression of the AhRR, too (1.1-fold ±0.1 of the control). Kaempferol did not affect the expression of this gene. Interestingly, the expression of AhRR mRNA seemed to be enhanced when either flavonoids were combined with 1 µM B[a]P (3.5-fold ±1.1 with 1 µM B[a]P + Q and 3.8-fold ±0.9 with 1 µM B[a]P + K of the DMSO level). However, Q or K showed a tendency to reduce the 10 µM B[a]P-induced AhRR expression (3.3-fold ±0.9 or 3.3-fold ±0.6 of the control, respectively).

The expression of ARNT seemed to be unaffected by B[a]P, the flavonoids or the combination of both. The AiP transcript was not modulated by B[a]P, either. However, quercetin alone trended to reduce the expression of the AiP mRNA. The combination of B[a]P + Q slightly reduced the transcription of AiP (0.8-fold ±0.1 of the control with 1 and 10 µM B[a]P + Q). On the other hand, kaempferol itself significantly reduced the AiP transcript (0.9-fold ±0.07 of the DMSO, fig. 3.14.a and b), whilst when combined with B[a]P this flavonoid did not show any effect.

While 1 µM B[a]P caused a 248-fold ±109 increase in CYP1A1 transcript compared with that of DMSO-treated cells, either flavonols alone showed no clear effect on the expression of this mRNA (fig. 3.14. c and d). 10µM B[a]P did not further increase the induction (315-fold ±175 of the control). Interestingly, the combination of B[a]P + Q slightly enhanced the CYP1A1 transcription in a concentration-dependent manner, whilst B[a]P + K showed a clear effect compared to that of 1 µM B[a]P (576-fold ±225 vs. 248-fold ±109). 10 µM B[a]P + 10 µM K caused a similar increase on CYP1A1 transcript (571-fold ±250 of the control).

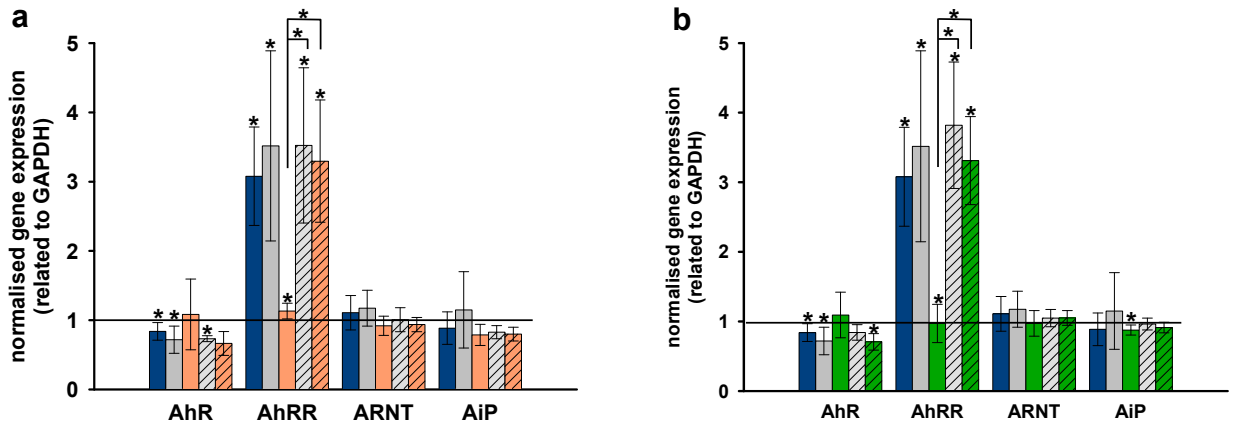
The PAH elicited in IEC-6 cells also a concentration-dependent increase in the amount of CYP1B1 mRNA by 4.6-fold ±2.1 with 1 µM and 6.4-fold ±2.8 with 10 µM B[a]P of the DMSO level. The flavonoids had no effect on CYP1B1 mRNA. B[a]P combined with the flavonoids showed a similar induction effects on the expression of CYP1B1 as observed also on the CYP1A1 mRNA. In a spite of no statistical significance, B[a]P + Q trended to enhance the CYP1B1 transcript further, reaching a maximum with 1 µM B[a]P + Q (7.3-fold ±0.4 of the control and 6.6-fold ±1 of the DMSO with 10 µM B[a]P + Q). Also, B[a]P + K showed a tendency to stronger induce the expression of CYP1B1, reaching a maximum of 8.3-fold

±0.005 of the DMSO level with 1 µM B[a]P + K and 7.1-fold ±0.7 of the control with 10 µM B[a]P + K.

As shown in fig 3.14.c and d B[a]P, quercetin and kaempferol alone did not influence the expression of Nrf2 and GCS mRNA. However, both genes were significantly induced when the carcinogen and kaempferol were combined in comparison to the amount of transcripts elicited by this flavonol alone. 1 µM B[a]P + K significantly enhanced the amount of GCS mRNA (~1.5-fold ±0.2 of the control) compared to 1µM B[a]P treated cells (0.9±0.4 of the control; 1.1±0.4 of the DMSO with 10 µM B[a]P). Also, B[a]P + Q significantly increased the amount the of Nrf2 transcripts compared to that of quercetin treated cells (~1.5-fold ±0.2 of the control). On the other hand, B[a]P-treated cells exhibit no increased Nrf2 expression (0.9±0.7 of the control with 1 µM B[a]P and 1.4±0.85 of the DMSO with 10 µM B[a]P).

In conclusion, B[a]P increased the expression of AhRR and CYPs. The amount of AhR transcript showed a slight concentration-dependent decrease by B[a]P. The other members of the pathways were unaffected by the PAH. Quercetin and kaempferol did not present a clear and strong effect on the gene expressions after 48 hours incubation. Quercetin caused a low but significant induction on the AhRR and CYP1B1 transcripts. On the other hand, kaempferol significantly reduced the expression of AiP. In the combination experiments, both flavonoids trended to a suppression of AhR and AiP (only by B[a]P + Q) transcription and a synergistic stimulation on the expression of CYP1A1, 1B1, Nrf2 and GCS in the IEC-6 cell line.

Influence of quercetin and kaempferol on the B[a]P-induced gene expression



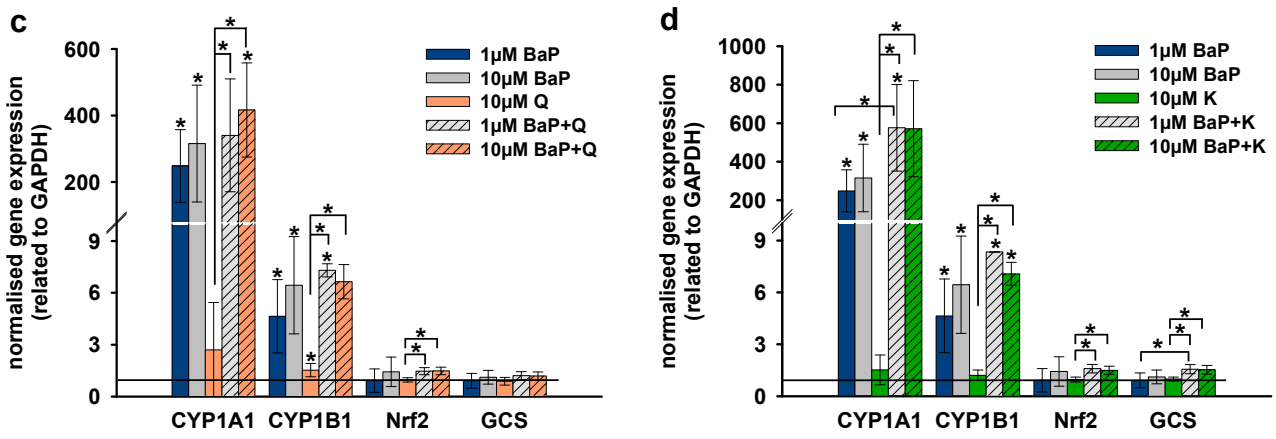


Fig. 3.14. Effect of quercetin and kaempferol on the B[a]P-induced gene expression.

Relative gene expression in IEC-6 cells after incubation with 1 or 10µM B[a]P, 10µM of each flavonoids or a combination of B[a]P plus either flavonoids. Gene expression of members of both pathway was measured by TaqMan® PCR. The gene expression was normalised to that of 0,1% DMSO and the relative fluorescence of the transcribed genes was related to that of the house-keeping gene GAPDH. The $2^{-\Delta\Delta Ct}$ method was used for the analysis. Results are expressed as M.V. ± S.D. ($n \geq 3$, $P < 0.05$). Values significantly different from the control are marked with an asterisk (One sample Student's t-test). An asterisk over a column shows a significant difference compared to DMSO-treated control cells whilst significant differences between the substances are shown above the bonds (unpaired Student's t-test).

3.2.5. Influence of quercetin and kaempferol on the B[a]P-induced protein expression

Chemicals can affect gene transcription, stabilise mRNA or induce a faster degradation of transcripts. Also, the amount of the protein does not necessarily correspond to the amount of its mRNA. Because of this, we looked for a possible modulation of the CYP1A1 and AhRR protein expression by means of western blot analysis.

IEC-6		DMSO	1µM B[a]P	10µM B[a]P	10µM Q	10µM K	1µM B[a]P+ 10µM Q	10µM B[a]P+ 10µM Q	1µM B[a]P+ 10µM K	10µM B[a]P+ 10µM K
AhR	MV	75,48	70,85	58,31	243,88	159,14	78,65	72,62	90,66	78,48
	SD	28,84	21,19	23,52	436,89	197,87	6,19	15,27	8,85	9,42
AhRR	MV	22,76	73,55	70,10	25,81	24,68	78,54	86,83	88,25	88,66
	SD	8,98	5,45	19,15	11,37	10,21	14,07	11,61	15,87	18,97
ARNT	MV	93,05	116,50	110,37	83,40	99,05	126,31	125,47	133,21	144,02
	SD	29,24	11,30	27,94	31,73	38,44	29,55	11,08	27,53	30,05
Nrf2	MV	396,76	718,15	659,40	385,38	432,31	732,77	883,55	810,16	919,93
	SD	148,27	82,87	179,02	132,34	186,58	236,28	202,96	266,69	200,69
CYP1A1	MV	0,04	8,66	9,10	0,09	0,04	7,53	10,83	12,25	14,79
	SD	0,01	2,25	4,08	0,11	0,02	5,39	3,62	7,29	6,12
CYP1B1	MV	46,47	380,21	359,00	93,99	56,29	513,11	480,62	587,29	476,70
	SD	22,37	88,41	125,84	55,47	27,05	25,23	63,61	13,09	31,98
GCS	MV	139,69	228,02	199,70	121,09	135,82	200,72	197,33	255,55	258,64
	SD	56,17	82,49	67,66	33,21	39,67	32,59	26,40	53,81	65,18
AiP	MV	61,52	54,04	63,02	47,99	57,33	51,24	54,39	59,91	63,36
	SD	12,08	6,56	11,01	3,33	9,66	8,95	3,45	5,05	6,24

Table 3.3. Relative transcript levels of the studied genes in IEC-6 cells related to the expression of GAPDH only (x1000). Note that the values are not normalized on the levels of control cells, i.e. they do not constitute induction factors such as in the figures shown before. Shown are mean values (MV) and standard deviations (SD).

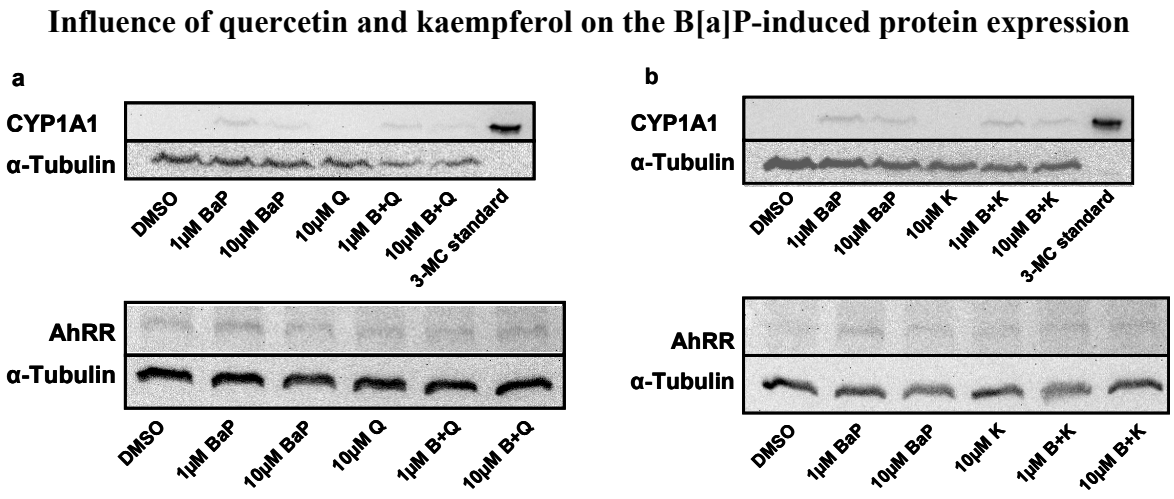


Fig. 3.15. Western blot analysis of CYP1A1 and AhRR protein expression in IEC-6 cells. IEC-6 cells were treated for 48 hours with B[a]P, flavonoids or the combination of both. Each lane was loaded with 50 µg of the total protein lysate. CYP1A1 and AhRR protein as well as α-tubulin were analysed by means of immuno-blotting.

As shown in fig. 3.15., faint bands of CYP1A1 protein were detected on western blots of treated IEC-6 cells. In comparison to the mRNA expression (see table 3.3.), DMSO-,

quercetin- and kaempferol-treated cells showed no detectable expression of the enzyme. Yet, B[a]P, B[a]P + Q and B[a]P + K-treated cells showed induction of this protein (fig 3.15.a). On the other hand, AhRR protein bands were detected in all samples. The inaccurate signals reached hardly the threshold of detection, making a semi-quantitative determination of the AhRR protein content difficult.

3.2.6. Effect of B[a]P, quercetin and kaempferol on the enzymatic EROD activity in IEC-6 cells

According to the low CYP1A1 mRNA and protein expression, IEC-6 cells revealed marginal EROD activity (fig. 3.16. and table 3.4.), which was considerably lower than in CaCo-2 cells (table 3.4.). However, 7-ethoxyresorufin is a well known substrate for the CYP1 family additionally including CYP1B1 and CYP1A2. As shown in table 3.3., the amount of CYP1B1 mRNA was appreciably higher than the amount of CYP1A1 mRNA in the IEC-6 cells. Noticeably, the amount of CYP1B1 protein (or an active form of this enzyme) was very low in comparison to its mRNA, as inferred by the marginal EROD activity present in these cells.

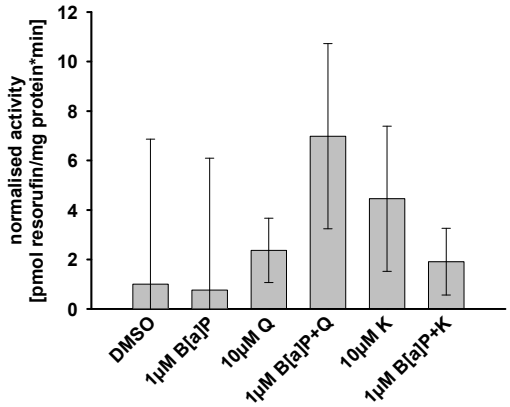


Fig. 3.16. Effect of quercetin or kaempferol on the B[a]P-induced CYP activity in IEC-6 cells. CYP activity after 48 hours incubation with 1 μM B[a]P, 10 μM of each flavonoids or in combination was detected by the EROD assay. The EROD activity is normalised to the DMSO-induced activity. Results are expressed as mean values ± S.D. ($n \geq 3$, $P < 0.05$.Unpaired Student's t-test).

IEC-6	DMSO	1μM B[a]P	Q	1μM B[a]P+ 10μM Q	K	1μM B[a]P+ 10μM K
MV	0,079	0,060	0,187	0,552	0,352	0,151
SD	0,463	0,421	0,103	0,296	0,232	0,107
CaCo-2	**DMSO**	**1μM B[a]P**	**Q**	**1μM B[a]P+ 10μM Q**	**K**	**1μM B[a]P+ 10μM K**
MV	0,580	6,372	6,139	6,036	0,553	3,491
SD	0,385	2,560	2,240	1,330	0,427	1,591

Table. 3.4. EROD activity in IEC-6 cells compared to CaCo-2 cells. Activities are expressed as pmol resorufin/mg protein*min (mean values ± S.D).

3.2.7. Gene expression profiling of B[a]P and flavonoid treated IEC-6 cells by microarrays

In addition to semi-quantitative TaqMan® Real time PCR analysis, we also analysed gene expression profiles in IEC-6 cells. Therefore, RNA of untreated and B[a]P-, quercetin and kaempferol treated IEC-6 cells was purified for microarray analysis. The analyses were performed by Laboratory for Genomics and Immunoregulation Program Unit Molecular Immune & Cell Biology LIMES (Life and Medical Sciences Bonn, University of Bonn) by using an Illumina-Chip (RatRef-12 Expression BeadChip) which provides insight into the RNA expression of 22,525 genes and offers hints of possible relevant signal pathways within the analysed cell line.

The results show that the number of differentially expressed genes, defined by an 1.5-fold up- or down-regulation, differs largely between B[a]P and the flavonoids. Interestingly, much more genes are influenced in their expression by quercetin compared to B[a]P:

	B[a]P (%)	**Q (%)**
differentially expressed genes	121 (0.54)	953 (4.23)
up-regulated genes	85 (0.38)	546 (2.42)
down-regulated genes	36 (0.16)	407 (1.81)

Differentially expressed genes modulated by B[a]P and quercetin compared to that of 0.1% DMSO control. The gene expression differences were analysis by variance analysis-ANOVA, listed are differences which reached >1.5-fold-changes vs. control, adjusted p-value <0.05.

The microarray show that CYP1A1 and CYP1B1 are among the first 5 strongest up-regulated genes by B[a]P-treatment of IEC-6 cells. Highest induction was found for Aldh3a1 which is also known to be regulated by the AhR but which was not further investigated here. Other regulated genes concern components of cell cycle regulation, transcriptional and translational control. These genes are not of direct concern for this work but the informations can be used for further studies on B[a]P and flavonoid effects in IEC-6 cells. In the discussion we mention a couple of analysed genes which could play a role in the modulatory regulation by B[a]P and flavonoids in this cell line.

3.3. Effects of B[a]P and flavonoids on HuTu-80 cells

3.3.1. Cytotoxicity of B[a]P and flavonoids on the HuTu-80 cell line

Although the HuTu-80 cell line derives from human duodenal carcinoma cells, it is rarely used as a model for studying physiological or pharmacological phenomena related to the small intestine. Consequently, there is a lack of pharmacological data regarding this cell line. Because of this, we had to gain some basic information on the cells. First, we determined non-cytotoxic concentrations of B[a]P and the flavonoids to be used for subsequent experiments. For this aim, we used the neutral red and BCA protein assays.

Effect of B[a]P, quercetin and kaempferol on HuTu-80 cell viability and protein content

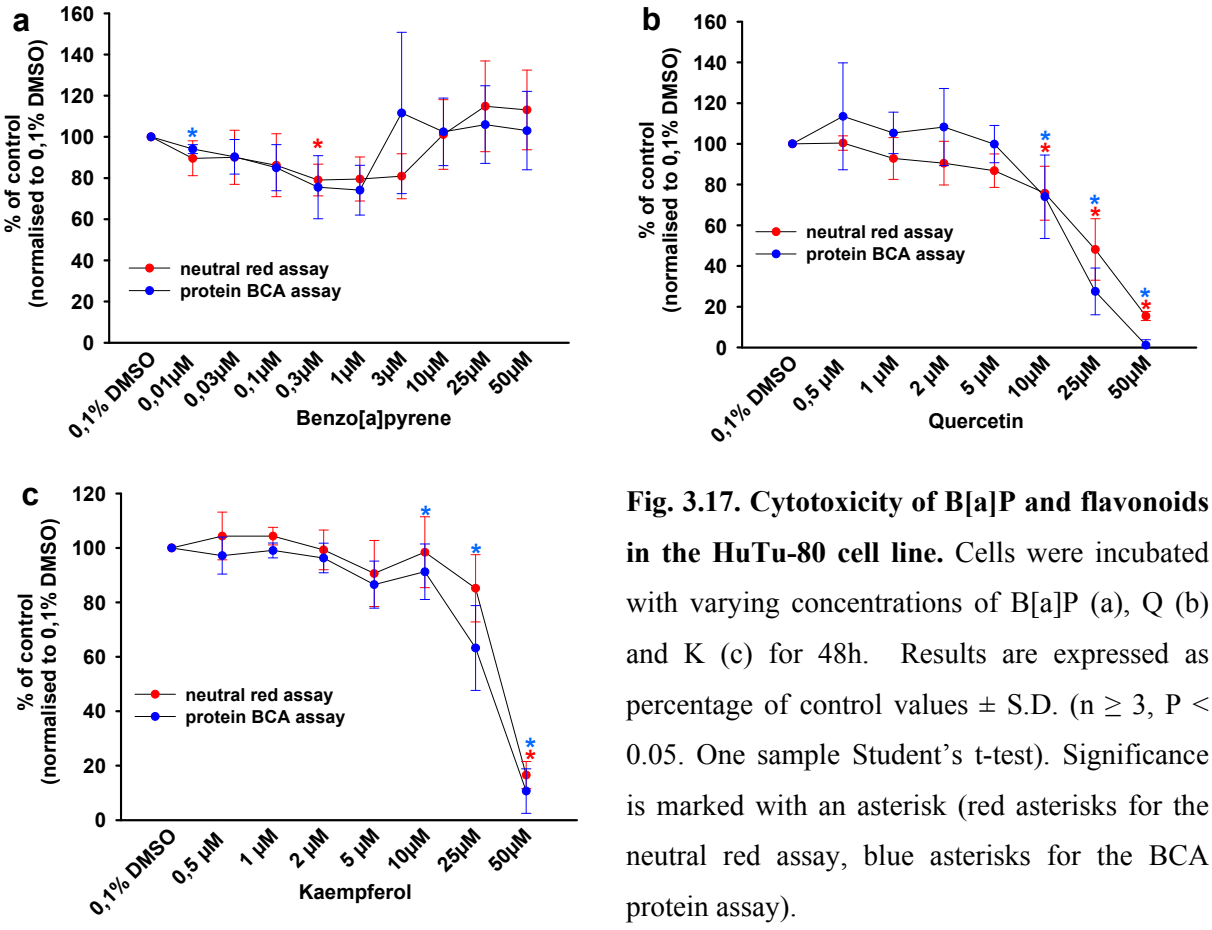


Fig. 3.17. Cytotoxicity of B[a]P and flavonoids in the HuTu-80 cell line. Cells were incubated with varying concentrations of B[a]P (a), Q (b) and K (c) for 48h. Results are expressed as percentage of control values ± S.D. ($n \geq 3$, $P < 0.05$. One sample Student's t-test). Significance is marked with an asterisk (red asterisks for the neutral red assay, blue asterisks for the BCA protein assay).

Similar to the results obtained with the other cell lines, the dose-response curve (fig. 3.17.a) evinced a slight but significant cytotoxic effect on the HuTu-80 cells elicited by B[a]P (viability sunk to 79.5±7.7 % and protein content to 75.5±15 % of the control after treatment with 0.3µM B[a]P). Cells exposed to 10 µM quercetin (Q) presented a clearly significant

decrease in the accumulation of the dye or in their protein content (75.7±13 and 74±20.5 % compared to the control, respectively). In contrast, 10 µM kaempferol (K) did not show a clear cytotoxic effect (viability of 98±13 % of the control) but a significant reduced protein content of 91±10 % of the DMSO control. After 48 hours exposure to 25 µM Q cell viability and protein content significantly sunk to 48 ±15 % and 27.5±11.5 % of the control, respectively. 25 µM K caused only a significant decrease on the protein content compared to 0.1 % DMSO treated cells (63±15.5 %) and the viability was not clearly reduced (74±30.8 % of the control). Compared to the other tested cell lines, HuTu-80 cells also showed cytotoxic effects after exposure to 50 µM of any of the flavonols (viability 15.5±2.2 % and 16.6±5 %; protein content 1.2±2.6 % and 10.7±8.1 % of the control value, with either quercetin or kaempferol, respectively).

With the combination 10 µM B[a]P + 10 µM Q or K, HuTu-80 cells showed no significant cytotoxic effects (cell viability 90.5±28.1 % and 100.2±8.3 %; protein content 95.4±41.1 % and 116±5.7 % of the control, respectively). An increase to 25 µM Q or K in the combination with 10 µM B[a]P, however, reduced the amount of living cells assessed by the NR assay to 12.5±5 % (B[a]P+Q) and 11.8±2.7 % (B[a]P+K) compared to the 0.1 % DMSO control. In a similar way, the protein content sunk to1.6±1.2 % (B[a]P+Q) and 3±6 % (B[a]P+K) of the control (fig 3.18.a and b). Also, this combination was as toxic as the positive control (3 % H_2O_2 for 48 hours).

Effect of B[a]P in combination with one of the flavonoids on the viability and protein content

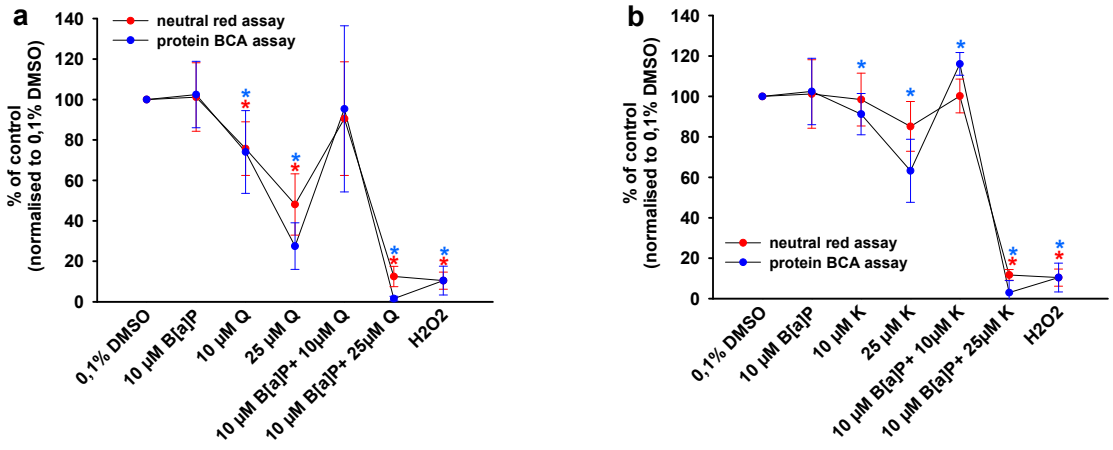


Fig. 3.18. Cytotoxicity of B[a]P in combination with quercetin or kaempferol in HuTu-80 cells. Cells were incubated with varying concentrations of benzo[a]pyrene combined with quercetin (a) or kaempferol (b) for 48h. Afterwards, the neutral red accumulation and BCA protein staining were measured. Results are expressed as percentage of control values ± S.D. ($n \geq 3$, $P < 0.05$; one sample Student's t-test). Values significantly different from the control are marked with an asterisk (red asterisks for the neutral red assay, blue asterisks for the BCA protein assay).

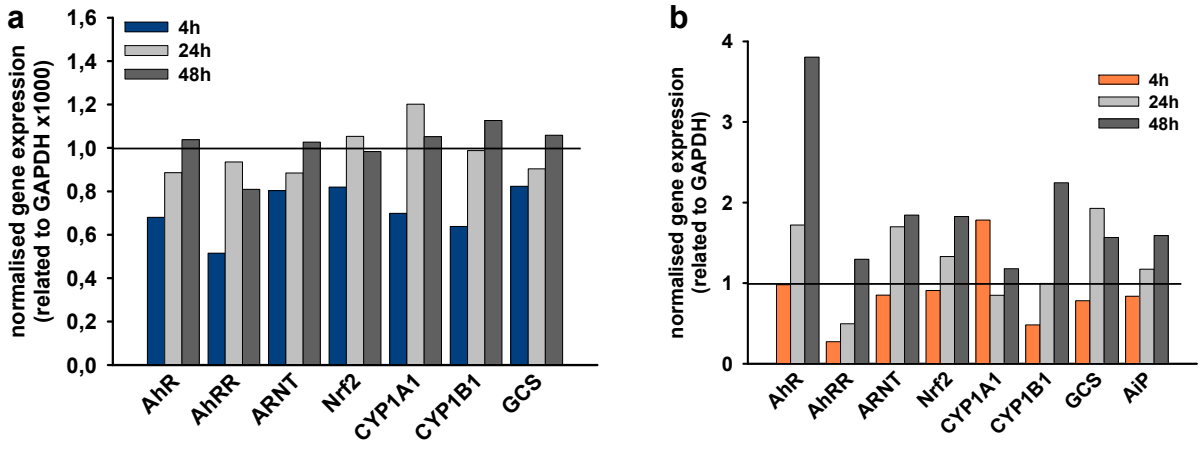
In conclusion, HuTu-80 cells showed a similar cytotoxic response to the substances as CaCo-2 and IEC-6. We were able to use B[a]P alone in a concentration up to 50 µM for 48 hours. The flavonoids alone could be used in a concentration of up to 25 µM. For the combination experiments only 10 µM B[a]P and 10 µM of the flavonols showed no cytotoxic effects.

3.3.2. Time-dependent gene expression pattern in HuTu-80 cells

We applied 10µM of each substance to study time-dependent effects on gene expression. After 4 hours of exposure, B[a]P trended to inhibit the expression of all detected genes which seemed to recover to the control level (0.1% DMSO) after 48 hours (fig. 3.19.a). 10 µM quercetin caused a time-dependent gene expression pattern (fig. 3.19.b). The expression of all genes at the 4 hours time-point showed a clear reduction and was increased at the later time-points. Nevertheless, CYP1A1 transcript seemed to be increased at the 4 hours measurement but recovered to the control level at the 48 hours time-point. In contrast, kaempferol caused a time-dependent decrease on AhRR, Nrf2, CYP1A1 and AiP transcripts. The amount of ARNT, CYP1B1 and GCS transcripts were increased by this flavonol at the 48 hours time-point. Only the expression of AhR was clearly induced at all time-points (fig. 3.19.c).

To retain comparability of all the experiments with the different cell lines, we decided to use 48 hours incubation with the substances in HuTu-80 cells, too.

Time dependent alterations in gene expression pattern in HuTu-80 cells in response to B[a]P and flavonoids



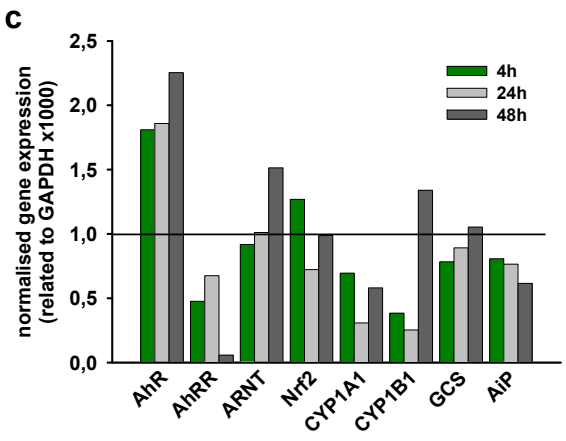


Fig. 3.19. Time dependent gene expression pattern after 48 hours incubation of B[a]P (a), quercetin (b) and kaempferol (c). HuTu-80 cells were treated with 10 µM of the indicated substances for 4, 24 and 48 hours. The mRNA of the members of the AhR- and Nrf2-pathways was determined by real-time TaqMan® PCR. The amount of transcripts was related to GAPDH mRNA and normalised to those of the DMSO treated control cells (set to 1). For the analysis the $2^{-\Delta\Delta Ct}$ method was used (n = 1).

3.3.3. Assessing effective concentrations of B[a]P, quercetin and kaempferol for studies with HuTu-80 cells

As shown in fig. 3.20.a, B[a]P did not reveal any clear effect on gene expression in the HuTu-80 cells after 48 hours incubation with all concentrations tested. The very low expression level of all the genes in this cell line (see table 3.5.) and the high variation in the results aggravates a statistical analysis. Also the responses of the cells to B[a]P exposure are rather low. Only the expression of CYP1B1 trended to be slightly induced by B[a]P while the amount of AhRR mRNA seemed to be reduced. Even higher concentrations of B[a]P (fig. 3.20.b) did not show a clear effect on the gene transcription.

HuTu-80 cells treated with 5 or 10 µM quercetin trended to increase the expression of almost all genes (~2-fold of the DMSO control). With the exception of CYP1B1 which was not affected (fig 3.20.c). 25 µM quercetin caused a induction on the gene expression compared to the transcription in B[a]P-treated cells, although the amount of the CYP1A1 and CYP1B1 mRNA in this experiment was reduced. Maximal induction of AhR, AhRR, ARNT and GCS by kaempferol was achieved at concentrations of 5 or 10 µM (~2-fold of the control level, fig. 3.20.e and f). An effect on the expression of the CYPs was not clearly observed by this flavonol.

Assessing effective concentrations of B[a]P, quercetin and kaempferol for HuTu-80 cells

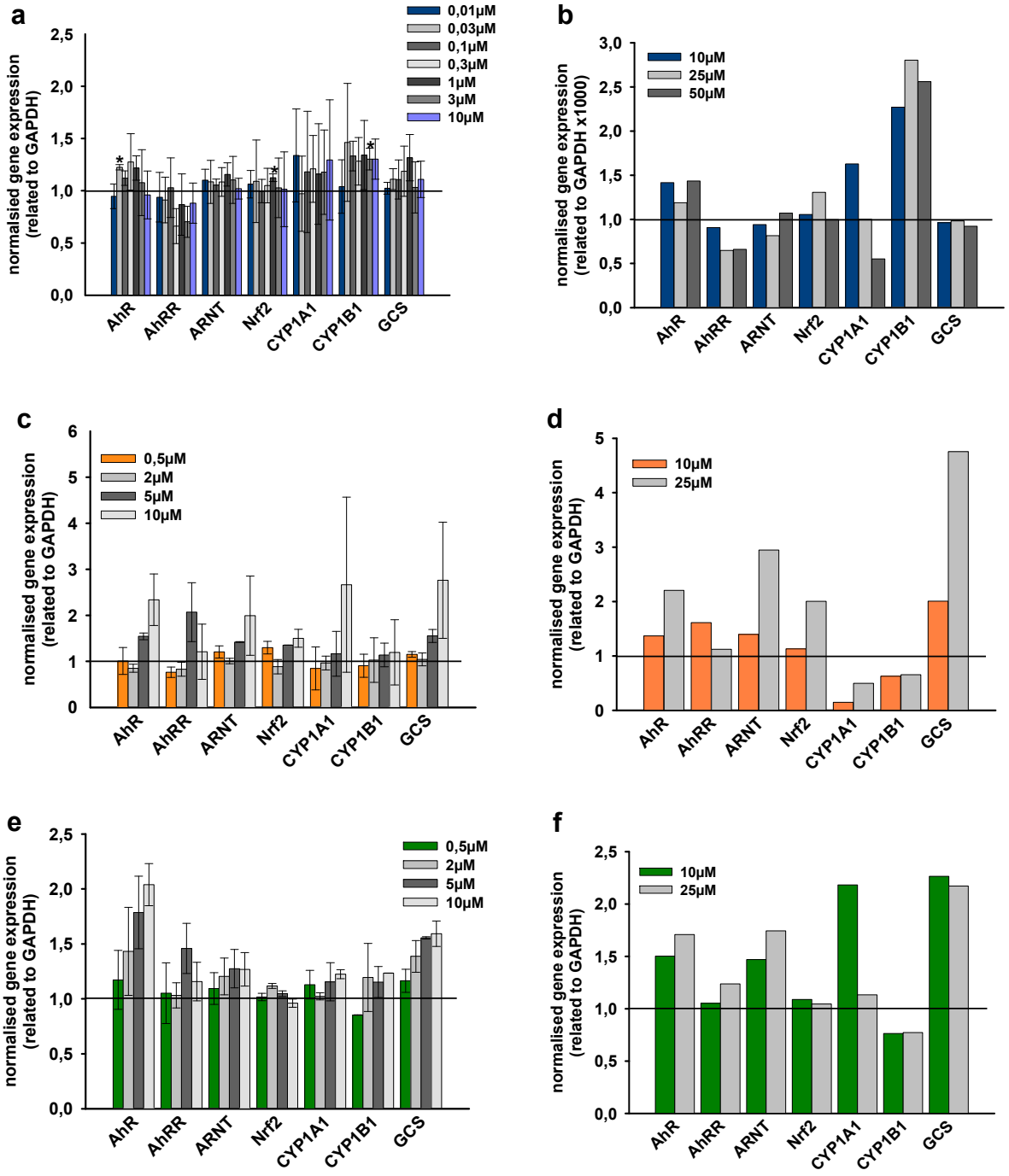


Fig. 3.20. Effect of B[a]P, quercetin or kaempferol on the gene expression pattern in HuTu-80 cells. Relative gene expression in HuTu-80 cells after incubation with different concentrations of B[a]P (a, b), quercetin (c, d) or kaempferol (e, f). Gene expression of members of both pathways was measured by real-time TaqMan® PCR. The gene expression was normalised to that of DMSO treated controll cells (set to 1) and the relative fluorescence was related to that achieved with the house-keeping gene GAPDH. The $2^{-\Delta\Delta Ct}$ method was used for the analysis (n = 1. Columns with error bars: n ≥ 2; one sample Student's t-test).

HuTu-80		DMSO	1µM B[a]P	10µM B[a]P	10µM Q	10µM K	1µM B[a]P+ 10µM Q	10µM B[a]P+ 10µM Q	1µM B[a]P+ 10µM K	10µM B[a]P+ 10µM K
AhR	MV	1,52	1,14	0,99	2,06	1,38	1,01	0,86	0,50	0,70
	SD	*0,87*	*0,50*	*0,41*	*1,01*	0,79				
AhRR	MV	0,04	0,02	0,03	0,04	0,03	0,04	0,02	0,01	0,02
	SD	*0,02*	*0,01*	*0,01*	*0,03*	*0,02*				
ARNT	MV	15,58	13,91	13,01	10,27	10,04	7,06	6,16	5,59	6,52
	SD	*8,95*	*4,58*	*5,87*	*4,96*	*5,89*				
Nrf2	MV	19,92	16,88	17,31	14,91	12,05	12,93	8,18	9,20	10,84
	SD	*10,60*	*7,62*	*7,58*	*5,54*	*4,48*				
CYP1A1	MV	0,030	0,029	0,038	0,009	0,022	0,016	0,017	0,021	0,014
	SD	*0,014*	*0,013*	*0,017*	*0,006*	*0,011*				
CYP1B1	MV	0,10	0,11	0,12	0,06	0,08	0,06	0,05	0,06	0,07
	SD	*0,05*	*0,05*	*0,05*	*0,05*	*0,06*				
GCS	MV	4,03	3,15	3,55	10,68	4,91	2,82	2,25	1,47	2,26
	SD	*2,24*	*1,31*	*1,46*	*14,96*	*3,42*				
AiP	MV	8,17	8,22	7,72	9,97	6,40	7,99	7,16	10,50	6,66
	SD	*0,15*			*4,16*	*0,67*				

Table 3.5. Relative values of gene expression data related to the expression of GAPDH (x1000). Note that the values are not normalized on the levels of control cells, i.e. they do not constitute induction factors such as in the figures shown before. Shown are mean values (MV) and standard deviations (SD).

With the exception of ARNT, Nrf2 and GCS genes, the very low gene expression level of the members of the AhR- and Nrf2-pathway prohibited us to draw any clear conclusions about the effects of B[a]P, quercetin and kaempferol in the HuTu-80 cells. Anyhow, B[a]P showed no clear effect on the gene expression of AhR and Nrf2 pathway members. On the other hand, quercetin and kaempferol seemed to induce slightly the expression of ARNT and GCS mRNA. Quercetin also increased the amount of Nrf2 transcript in the HuTu-80 cell line.

3.3.4. Influence of quercetin and kaempferol on the B[a]P-induced gene expression

Since HuTu-80 cells showed a very low expression of the genes of the AhR and Nrf2 pathways, we tested the cells only once with combinations of B[a]P and each flavonol only in order to check whether the cells respond to the combination by possible synergistic effects. All measurements concerning B[a]P or flavonoid-dependent effects on gene expression are summarised in the figures 3.21. and 3.22.

As already shown, B[a]P did not modify clearly the expression of any gene of both pathways. Quercetin alone significantly induced the amount of Nrf2 and GCS transcripts, while this

flavonol trended to increase the expression of ARNT and AhR. In addition, kaempferol trended to induce the expression of AhR, ARNT, CYP1A1 and GCS mRNA (fig. 3.21.a and fig. 3.22.). In the combination experiment, we could observe a tendency of inhibition of both flavonoids on the ARNT transcript. However, the expression of Nrf2 showed stimulation by kaempferol or to reach a similar induction as in the quercetin-treated cells.

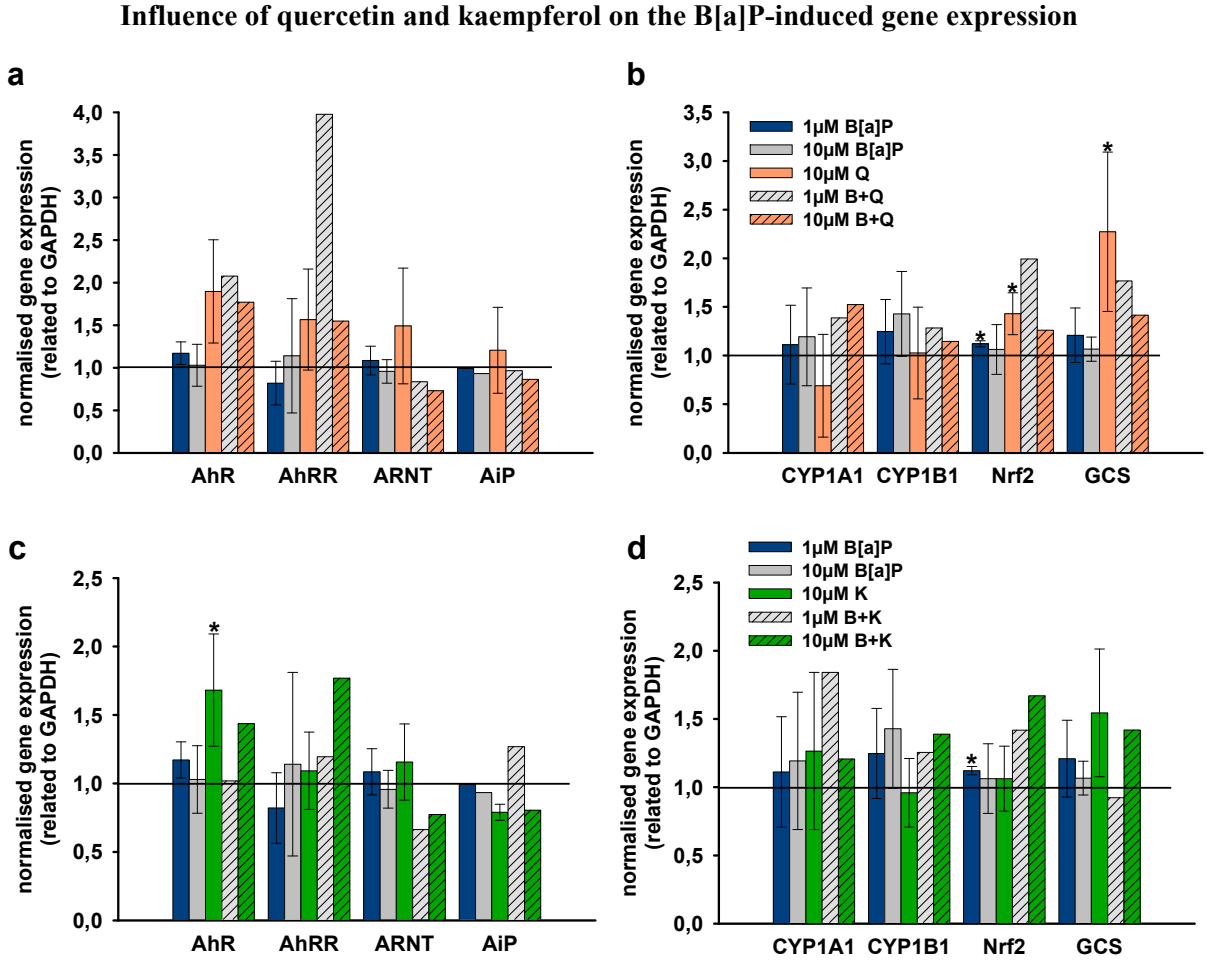


Fig. 3.21. Effect of quercetin and kaempferol on the B[a]P-induced gene expression.

Relative gene expression in HuTu-80 cells after incubation with 1 or 10µM B[a]P, 10µM of each flavonoids or a combination of B[a]P plus either flavonoid. Gene expression was measured by TaqMan® PCR. Expression levels were related to that of the house-keeping gene GAPDH and normalised to that of DMSO treated control cells. The $2^{-\Delta\Delta Ct}$ method was used for the analysis. Results are expressed as means ± S.D. ($n \geq 3$, $P < 0.05$ single substances experiments; columns without error bars: n = 1). Values significantly different from the control are marked with an asterisk (one sample Student's t-test).

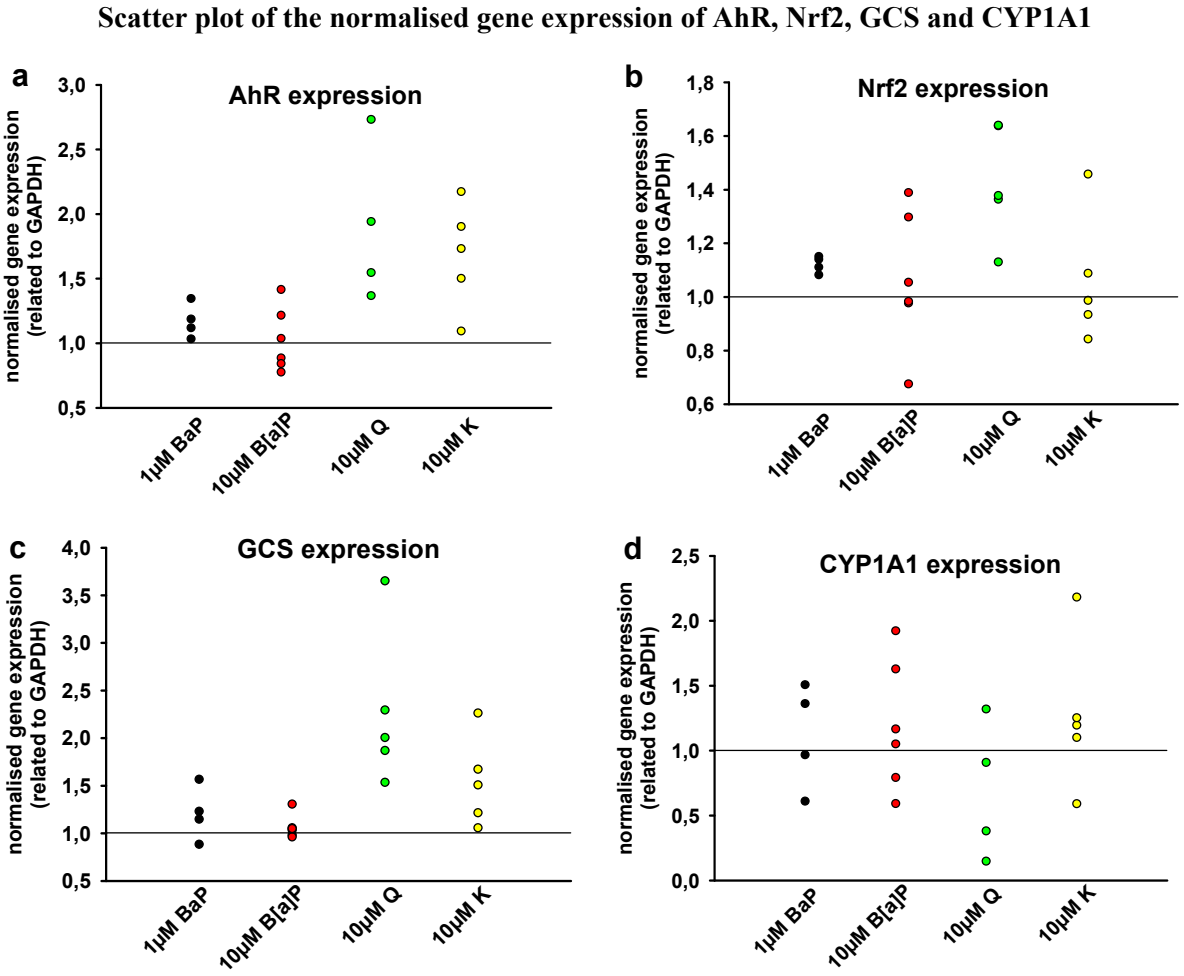


Fig. 3.22. Scatter plot diagram of AhR, Nrf2, GCS and CYP1A1 expression influenced by B[a]P, quercetin and kaempferol in HuTu-80 cells. Each point represents the mean value of at least three normalised measurements of at least four experiments.

Influence of quercetin and kaempferol on the B[a]P-induced protein expression

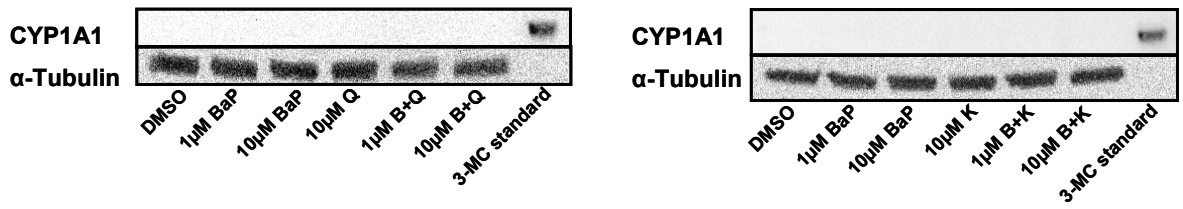


Fig. 3.23. Western blot analysis of CYP1A1 protein expression in the HuTu-80 cell line. HuTu-80 cells were treated for 48 hours with B[a]P, flavonoids or the combination of both. Each lane was loaded with 50 µg of the total protein lysate. CYP1A1 protein as well as α-tubulin were analysed by means of immuno-blotting.

In line with the very low transcript level, we could not detect any CYP1A1 protein by means of western blots analysis (fig. 3.23.) as well as any EROD activity.

3.4. Comparison between duodenum and colon derived cell lines

3.4.1. Differences in the AhR-/ Nrf2-pathway ratio between duodenal and colon derived tested cell lines

To detect a possible difference in the mechanism of the metabolic response in the tested cell lines, we calculated the ratio of the mRNA levels of the receptors Nrf2 and AhR and of their specific target genes GCS, CYP1A1 and CYP1B1.

As table 3.6. shows, the ratio Nrf2/AhR in CaCo-2 cells was unaffected by the tested substances, with higher amounts of AhR mRNA compared to Nrf2 mRNA. Interestingly, both duodenal cell lines, IEC-6 and HuTu-80, showed a higher constitutive level of Nrf2 than of AhR mRNA in comparison to CaCo-2 cells. While in HuTu-80 cells 10 µMB[a]P induced a shift in favour of the Nrf2 transcript as a result of a significant induction on the transcription of this gene (Nrf2/AhR ratio 17.5; see fig. 3.24.a and 3.21.b), in IEC-6 cells this shift was due to the significant inhibition on the transcription of AhR mRNA (Nrf2/AhR ratio 11.3 see fig.3.24.a and 3.14.a). Although the flavonoids increased the amount of AhR mRNA in both duodenal cell lines, yet the level of Nrf2 transcript was higher. In HuTu-80 cells, quercetin alone increased the transcription of AhR, decreasing the Nrf2/AhR ratio to 7.2, whilst when combined with 10 µM B[a]P this ratio seemed to revert to 9.5, perhaps due to a partially agonist effect of the flavonoid. However, B[a]P + Q did not modify the Nrf2/AhR ratio in IEC-6 cells compared to B[a]P alone. In both duodenal cell lines, kaempferol increased the AhR transcript, whilst B[a]P plus kaempferol did not show any clear partial agonist effect (fig. 3.24.b). Thus, the Nrf2-pathway seems to play a more important role in the duodenal cells compared to the colon derived cell line.

3.4.2. Difference in the GCS/CYP ratio between duodenal and colon derived tested cell lines

In CaCo-2 cells, we observed a notably lower GCS/CYP1A1 ratio in comparison to the duodenal derived cell lines (20.7 for CaCo-2, compared to 132.7 and 3885.5 for HuTu-80 and IEC-6 cells, respectively). The expression of CYPs was strongly increased by either B[a]P,

quercetin and the combination of B[a]P plus each of both flavonoids. Kaempferol alone did not increase the level of CYPs mRNA.

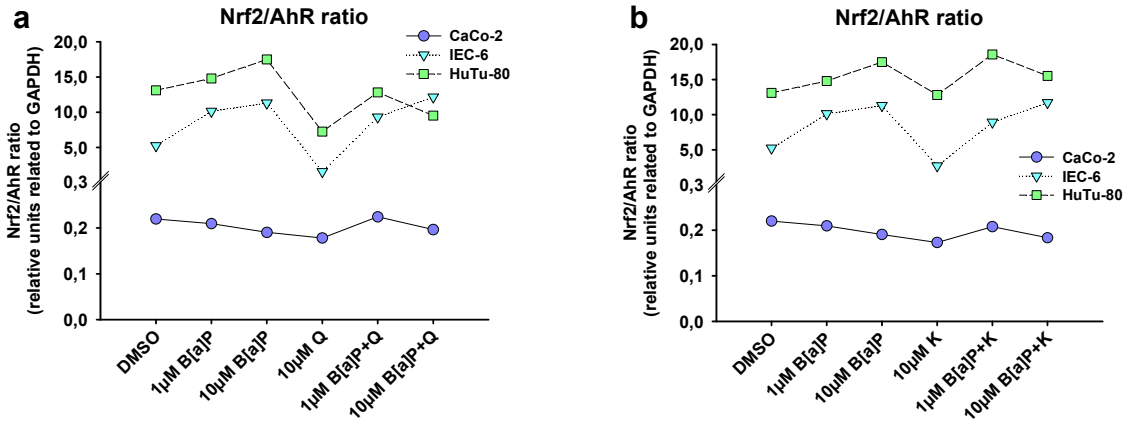
3.4.3. Difference in the expression of CYP1A1 and CYP1B1 between duodenal and colon derived tested cell lines

It is possible that CYP1A1 plays a more important role in CaCo-2 cells than CYP1B1 does. For example, the GCS/CYP1A1 ratio drops from 20.7 (with DMSO) to 0.3 (with 10 µM B[a]P; i. e. a factor of 69), whilst the GCS/CYP1B1 ratio sunk from 114.4 (with DMSO) to 4.1 (with 10 µM B[a]P; i.e. a factor of 28; fig. 3.24.c and d). IEC-6 and HuTu-80 cells showed a clear increase of CYP mRNAs induced by B[a]P or in combination with each flavonoid.

		DMSO	1µM B[a]P	10µM B[a]P	10µM Q	1µM B[a]P+ 10µM Q	10µM B[a]P+ 10µM Q	10µM K	1µM B[a]P+ 10µM K	10µM B[a]P+ 10µM K
Nrf2/AhR	CaCo-2	0,22	0,21	0,19	0,18	0,22	0,20	0,17	0,21	0,18
	IEC-6	5,26	10,14	11,31	1,58	9,32	12,17	2,72	8,94	11,72
	HuTu-80	13,11	14,80	17,49	7,24	12,82	9,51	8,74	18,58	15,52
GCS/CYP1A1	CaCo-2	20,67	0,59	0,31	0,32	0,22	0,24	18,81	0,56	0,33
	IEC-6	3885,48	26,33	21,94	1378,19	26,67	18,21	3732,58	20,86	17,49
	HuTu-80	132,70	108,13	93,96	1176,87	178,90	130,33	223,61	70,33	165,08
GCS/CYP1B1	CaCo-2	114,36	12,09	4,14	10,57	5,88	4,68	57,01	13,77	4,22
	IEC-6	3,01	0,60	0,56	1,29	0,39	0,41	2,41	0,44	0,54
	HuTu-80	41,59	27,98	29,79	172,71	46,11	41,33	59,97	24,61	34,20

Table 3.6. Transcription factor and target gene expression ratios in the different cell lines.

Gene expression ratio after 48 hours in CaCo-2, IEC-6 and HuTu-80 cell line



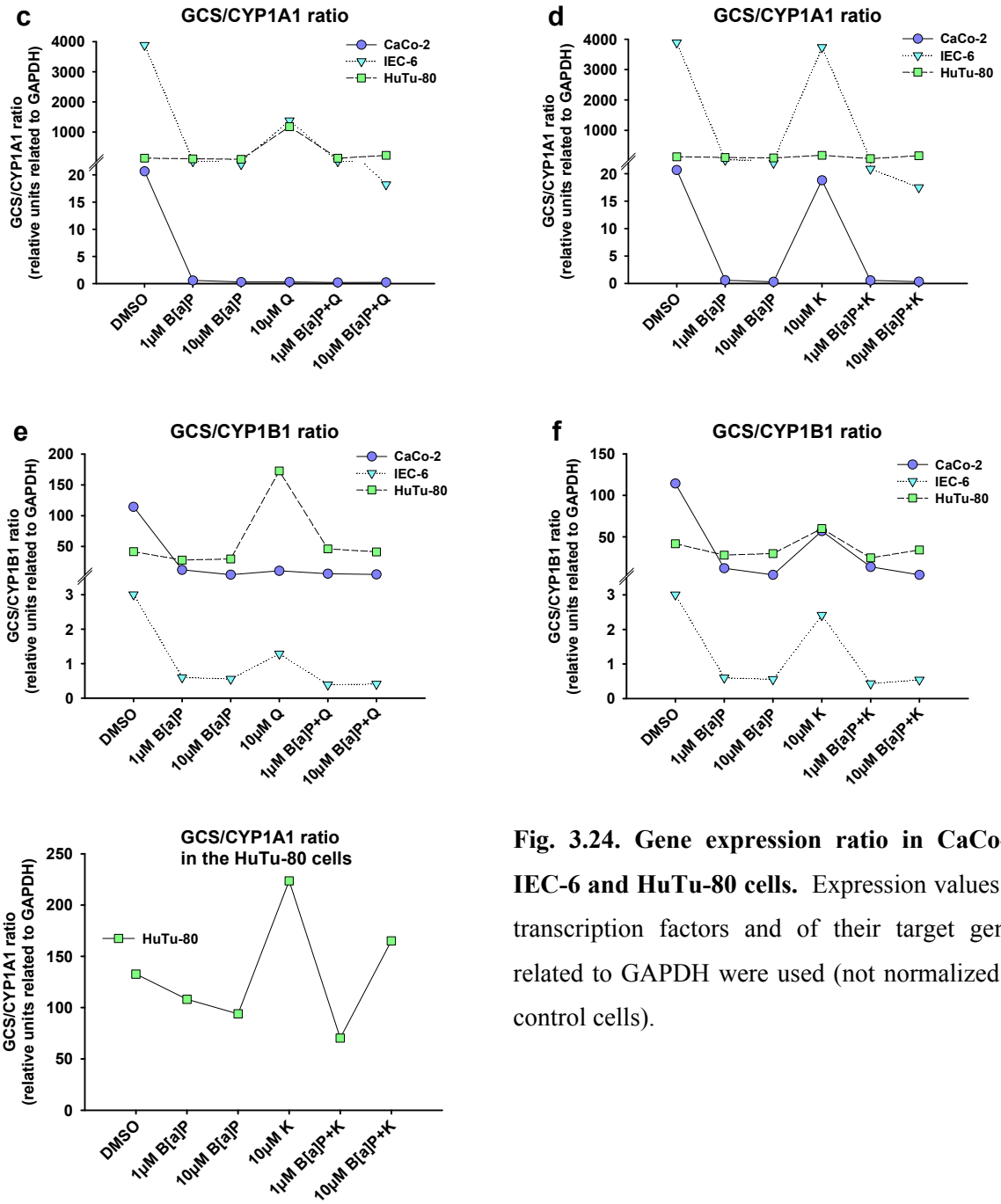


Fig. 3.24. Gene expression ratio in CaCo-2, IEC-6 and HuTu-80 cells. Expression values of transcription factors and of their target genes related to GAPDH were used (not normalized to control cells).

Noticeably, CYP1B1 was the dominant transcript compared to CYP1A1 in both duodenal cell lines (CYP1B1 = 0.1 and CYP1A1 = 0.03 in HuTu-80 cells; CYP1B1 = 46.5 and CYP1A1 = 0.04 in IEC-6 cells). IEC-6 cells exhibit a very strong increase in the expression of CYP1B1 by the substances (e.g. GCS/CYP1B1 ratio sunk from 3 in DMSO treated control cells to 0.6 in cells treated with 10 µM B[a]P). However, CYP1B1 mRNA did not seem to be translated to result in an equal protein level (fig. 3.24.e and f). On the other hand, GCS is a target gene of the transcription factor Nrf2 and is not affected by AhR (Kampkötter A., personal

communication). Interestingly, the duodenal derived cell lines showed a constitutive higher amount of GCS mRNA (see above).

3.5. Effect of ethoxyquin and *tert.*-butylhydroquinone on the gene expression in IEC-6, CaCo-2 and HuTu-80 cell lines

To appreciate the modulatory activity of the flavonols on the B[a]P-induced affects we tried to distinguish the gene expression pattern in both cell lines using the selective Nrf2 activator ethoxyquin (EQ) and the AhR/Nrf2 activator *tert*-butylhydroquinone (tBHQ) as described by C. Köhle et al. 2006 and W. Miao et al. 2004. We incubated both cell lines for 48 hours with 2.5 and 12.5 µM EQ as well with 2 and 10 µM tBHQ (W. Miao et al. 2004). Afterwards, we determined the mRNA levels of the examined genes by real time-RT-PCR.

Effect of ethoxyquin and *tert.*-butylhydroquinone on the gene expression in CaCo-2, IEC-6 and HuTu-80 cell lines

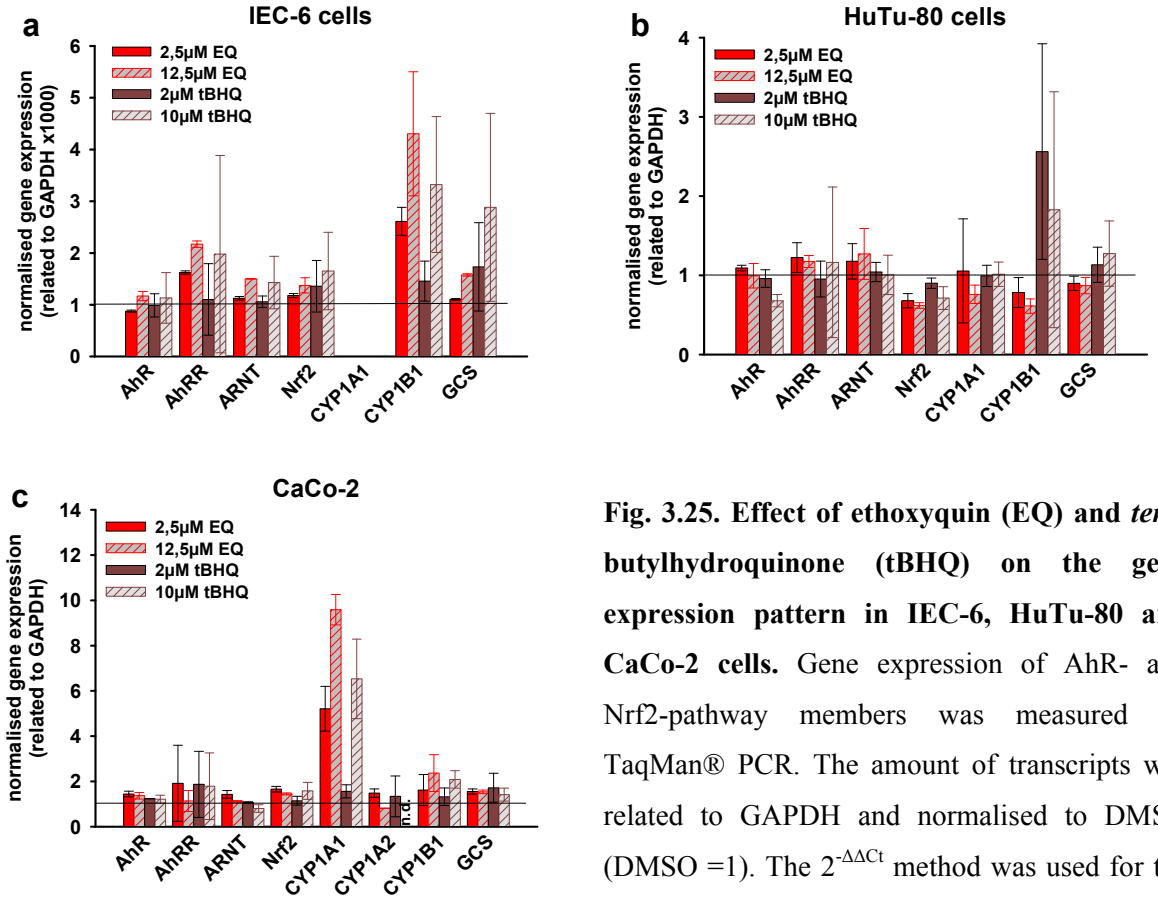


Fig. 3.25. Effect of ethoxyquin (EQ) and *tert.*-butylhydroquinone (tBHQ) on the gene expression pattern in IEC-6, HuTu-80 and CaCo-2 cells. Gene expression of AhR- and Nrf2-pathway members was measured by TaqMan® PCR. The amount of transcripts was related to GAPDH and normalised to DMSO (DMSO =1). The $2^{-\Delta\Delta Ct}$ method was used for the analysis. Results are expressed as means ± S.D. ($n \geq 1$) Each column represents the mean value of at least two normalised measurements.

In IEC-6 cells, EQ induced clearly the expression of AhRR and CYP1B1 in a concentration-dependent manner. Likewise, the transcripts of GCS, ARNT and Nrf2 were slightly increased. In HuTu-80 cells, this antioxidant trended to decrease the expression of Nrf2, GCS and CYP1B1. In the CaCo-2 cell line, the CYP1A1 transcript was strongly increased in a concentration-dependent manner by ethoxyquin, whilst Nrf2, GCS and CYP1B1 only trended to be enhanced. Comparatively, the AhR/Nrf2 activator *tert.*-butylhydroquinone trended to increase the expression CYP1B1 in the duodenal cell lines and CYP1A1 in CaCo-2 cells. In HuTu-80 cells, tBHQ trended to reduce also the AhR transcript, however, the other genes of the pathway seemed to be unaffected by this antioxidant in the three cell lines.

4. Discussion

Colorectal carcinomas are the third most common form of cancer and the second leading cause of cancer-related death in the Western world with 655,000 deaths worldwide per year; www.who.int/mediacentre/factsheets/fs297/en/). In comparison, there were only 2,750 cases of duodenal carcinoma reported to the National Cancer Database from 1985 to 1995. It is postulated that a major cause for these malignancies is a diet rich in fat, refined carbohydrates and animal protein, combined with low physical activity. Genetic susceptibility appears to be involved in less than five per cent of cases. The WHO World Cancer Report (2003) provides clear evidence that action on smoking, diet and infections can prevent one third of cancers and another third could be cured.

Plant secondary metabolites like flavonoids have biological activity that may be beneficial to health. For instance, these polyphenolic compounds scavenge free oxidative radicals and pose antioxidant, anti-thrombotic and anti-carcinogenic activities. The small intestine, as the first site of exposure to xenobiotics, is an important site of metabolism of flavonoids.

4.1. The role of the AhR- and Nrf2-pathways in duodenum

The aryl hydrocarbon receptor (AhR) pathway plays an important role in developmental, physiological and detoxification functions. Phase-I enzymes like CYP-1A1, -1A2 and -1B1 are target genes of the AhR receptor pathway. The transcription factor NF-E2-related factor-2 (Nrf2) is known as to be very effective in protecting the cell against oxidative/electrophile stress. Its target genes are phase II enzymes like NQO1 (NADPH dehydrogenase quinone-1), GST (gluthation-S-transferase) and UGT (UDP-glucuronosyltransferase). These two pathways are functionally connected. W. Miao et al. (2005) demonstrated that Nrf2 gene transcription is directly modulated by AhR activation. In the gastrointestinal tract AhR–Nrf2 interaction may facilitate detoxification by efficiently coupling Phase I and II xenobiotic-metabolising enzymes (C. Köhle et al., 2006; B. Ebert et al., 2006).

In duodenal epithelial cells dietary assimilated PAH (polycyclic aromatic hydrocarbons; e.g. benzo[a]pyrene; B[a]P) activates the AhR-pathway and causes an increase in the expression of CYP1A1 and CYP1B1 (P.H. Roos et al., 2002; A. Quaroni et al., 1981). CYP1A1 is known to metabolise B[a]P to a reactive epoxid which induces cancer by forming DNA adducts. Animal studies have shown that dietary intake of B[a]P causes increased incidence of

tumours at several sites, particularly in the gastrointestinal tract (A.J. Triolo et al., 1977). A fast detoxification of reactive metabolites by phase II enzymes is important to avoid DNA-adduct formation and subsequent cancer development. The coupled AhR and Nrf2 gene batteries could play an important role in the duodenum and it could be the reason for the lower cancer risk in this intestinal region.

Dietary phytochemicals like the flavonoids quercetin and kaempferol have a positive influence on the detoxification of carcinogens and provide protection against chemical carcinogenesis (Y. Moon et al., 2005; R. Cermak et al., 2006; M. Murray et al., 2006). Flavonoids are mostly present as glycosides in which one or more carbohydrate groups are bound to phenolic groups by glycosidic linkage (K. Murota et al., 2003). Even though glycosylated flavonoids could be absorbed in the small intestine, the deglycosylated fraction is absorbed more efficiently in the duodenum after de-conjugation by β-glucuronidase (A.J. Day et al., 1998; K. Murota et al., 2002). Once in cytosol, the flavonoids interact with transcription factors like AhR and Nrf2, among others.

4.2. Duodenal cell line models

First, the molecular background and the limitations of duodenal cell line models will be discussed. In our experiments we showed a high expression of CYP1A1 in CaCo-2 cells. Both, CYP-1A2 and -1B1 were constitutively expressed and further induced by B[a]P and quercetin. CYP1A1 protein was clearly detected by immunoblots and we observed a B[a]P- and quercetin-induced CYP activity by means of the EROD assay. Thus, CaCo-2 cells proved to be a suitable tool to characterise the modulatory effects of B[a]P, quercetin and kaempferol on the AhR-dependent gene and protein expression. However, these cells differed in their gene and protein expression profile from duodenal cells.

CaCo-2 monolayers are often used as a human model for intestinal absorption and metabolism. This human colon derived cell line morphologically resembles small-intestinal cells because they also present typical small-intestine micro-villous hydrolases and nutrient transporters in culture for longer periods (H.P. Hauri et al., 1985; M. Rousset et al., 1989; S. Howell et al., 1992). However, the limitations of the CaCo-2 cell model have been already demonstrated in several publications. For example, there are significant differences in gene expression patterns of phase I and II enzymes between CaCo-2 cells and human duodenal

enterocytes (D. Sun et al., 2002). Additionally, N. Petri et al. (2003) showed that CaCo-2 cells had a significantly lower LPH (lactase-phlorizin hydrolase) activity compared to that of the human small intestine. Another difference is the expression of CYP3A4, which is the predominant isoform in the small intestine (Q. Zang et al., 1999; M.F. Paine et al., 1997; D. Sun et al., 2002; M.F. Paine et al., 2006), whilst in CaCo-2 cells the expression of this enzyme is by far lower (A. Lampen et al., 1998 and D. Sun et al., 2002). RT-PCR of human duodenal enterocytes revealed the expression of CYP1-A1, -1B1, -2C, -2D6, -2E1, -3A4, and -3A5 mRNAs. Comparatively, CYP-1A2, -2A6, -2A7, -2B6, -2F1, -3A7, and -4B1 mRNAs were not detected. Interestingly, when probed by immunoblots, only CYP3A4 and -2C proteins were detected whilst CYP-1B1, -2E1, -2D6, and -3A5 proteins were not (L.S. Kaminsky et al., 2003; X. Ding et al., 2003). CYP1A1 protein or catalytic activity was undetectable or very low (K.F. Windmill et al., 1997; K.S. Lown et al., 1997; P.G. Pearson and L.W. Wienkers, 2008), and its expression was probably inducible rather than constitutive (J. Buchthal et al., 1995; M.F. Paine et al., 1999).

We also showed that duodenal HuTu-80 cells constitutively expressed CYP-1B1 and -1A1 mRNA and that B[a]P and quercetin did not increase their expression. Compared to CaCo-2 cell line, HuTu-80 cells exhibited very low levels of CYP1A1 transcripts, so that CYP1A1 protein could not be detected even in B[a]P-treated cells. Correspondingly, we could not detect any EROD activity in this human duodenal cell line, which is another difference when compared to the human duodenum *in vivo*. In line with our results, A. Lampen et al. in 1998 demonstrated that human HuTu-80 and rat IEC-6 duodenum derived cell lines show neither CYP-1A1, -1A2 and -1B1 enzymatic activity nor gene or protein expression.

Our results show that in contrast to the HuTu-80 cells IEC-6 cells constitutively expressed CYP-1B1 and -1A1 mRNA and that B[a]P and quercetin increased their expression. L.S. Kaminsiky et al. (2003) described that CYP1A1 is not constitutively expressed *in vivo* in the rat small intestine, but is the most prominent inducible CYP isoform. Also, treatment of rats with phenobarbital/naphtoflavon induced CYP-dependent EROD activity in the rat duodenum (N. Hernández-Martínez et al., 2007). Also, P.H. Roos et al. (2002 and 2004) demonstrated that PAH induced the expression of CYP1A1 in rat and minipig duodenal cells. In our experiments, the duodenal rat cell line showed a slight CYP1A1 protein expression but no CYP activity. In contrast to the in vivo situation, CYP1B1 and not CYP1A1 seemed to be the

predominant CYP isoform in this cell line (L.S. Kaminski et al., 2003; D. Mitschke et al., 2008).

4.3. Cytotoxicity of B[a]P, quercetin and kaempferol on the intestinal cell line

Benzo[a]pyrene is a well known AhR ligand and the susceptibility to B[a]P mediated CYP induction varies strongly in different cell lines. On the other hand, it is already known that the passage number of cell lines is an important factor which affects the cell response to diverse substances (D.W. Hamilton et al., 2005; B. Ebert et al., 2007). As a human intestine model, CaCo-2 cells are often used for studying the effect of compounds present in the diet such as xenobiotics like TCDD (2,3,7,8-tetrachlordibenzodioxin) and phytochemicals like quercetin and kaempferol. For our experiments we used CaCo-2 cells at passages from 39 to 48 and HuTu-80 cells at passages 31 to 38. Unfortunately, the provider was not able to give us information about the number of passages of the IEC-6 cells, as also stated by a number of other authors (G. Agullo et al., 1994; S. Kuo et al., 1996; C. Pohl et al., 2006).

By means of the accumulation of the neutral red dye we did not observe any cytotoxic effect of 50 µM B[a]P after 48h incubation in CaCo-2 cells. Consistent with our results, N. Sugihara et al. (2007) did not observe a cytotoxic effect in these same cells after 72h incubation with 50 µM B[a]P as judged by the MTT method. Comparatively, in RTL-W1 cells (fish liver cell line) K. Schirmer et al. (2000) showed a small but significant decline in cell viability after 48h exposure to 10 µM B[a]P, whilst B.D. Jeffy et al. (2000) showed that 1 µM B[a]P was cytotoxic in MCF-7 cells after 72h incubation.

To our knowledge, IEC-6 and HuTu-80 cell lines have not been tested for cell survival after incubation with B[a]P. As described for CaCo-2 cells, B[a]P exerted no cytotoxic effect after 48h incubation.

Flavonoids are the most abundant phytochemicals in our diet. Among these, quercetin and kaempferol are often used for studying the effects of flavonols on different pathways and cell mechanisms. Cytotoxic effects of these substances have been analysed. The use of different cell lines and different methods renders it difficult to select a non-cytotoxic concentration of them for our the own investigations (e.g. Q. Zang et al., 2009; V. Soares et al., 2006; E.H. Rodgers et al., 1998; L. Valerio J., 2001).

In CaCo-2 cells we observed a strong cytotoxic effect of quercetin and kaempferol (25 µM) after 48h incubation. Also, C. Pohl et al. (2006) and S. Kuo et al. (1996) observed a significant cytotoxic effect of 50 µM quercetin (IC_{50} = 45 µM) after 48h of exposure. Accordingly, G. Agullo et al. (1994) showed that 15 µM quercetin caused a rapid decrease in lactate release and a fall in the cellular ATP concentration after 48 hours exposure. Comparatively, M.J. Van Erk et al. (2005) did not see cytotoxic effects of quercetin (5-50 µM) after 48h incubation in these same cells, as judged by trypan blue accumulation.

HuTu-80 cell line is rarely used for toxicological studies in particular as a model for studying the AhR- or Nrf2-pathway, so that only limited data on cytotoxicity are available. For instance, M.L. Ackland et al. (2005; the only paper we found regarding this cell line and cytotoxicity) described that after 72 hours treatment with 5 µM quercetin or 10 µM kaempferol the HuTu-80 cell proliferation was significantly reduced, with a stronger effect exerted by quercetin than by kaempferol. Accordingly, we showed that quercetin caused a concentration-dependent reduction on cell survival after 48 hours incubation. Additionally, 50 µM kaempferol strongly reduced the cell viability after 48 hour treatment, too, although this effect was not as strong as with quercetin. Interestingly, the cytotoxicity on CaCo-2, IEC-6 and HuTu-80 cells showed a similar susceptibility to B[a]P, quercetin and kaempferol. In regard to IEC-6 cells, we showed that after 48 hours of exposure to 50 µM kaempferol almost 90 % of the cells died. A similar effect was observed by S. Kuo et al. (1996). It is important to note that in the *Salmonella typhimurium* test quercetin is genotoxic and that kaempferol is hydroxylated to quercetin by CYP1. That means that non-toxic flavonoids which are metabolised by CYPs could exhibit cytotoxicity at a later time point (R. Arroo et al., 2009).

In our experiments, the combination 10 µM B[a]P plus 25 µM of each flavonol showed the strongest cytotoxicity effect on the treated cells. In sharp contrast to our results, K. Hyun-Jung et al. (2000) observed in HepG2 cells (human hepatocellular carcinoma) that 51.7 µM quercetin and 42.7 µM kaempferol reduced the toxicity induced by B[a]P. However, it must be noted that these authors pre-incubated the cells with varying concentrations of flavonoids for 18 h. The cells were then incubated for another 72 h with either flavonoids and 4 µM B[a]P. It must also be pointed out that HepG2 cells seem to be susceptible to B[a]P. For instance, S.Y. Park et al. (2006) showed that after 24 hours even very low concentrations of this PAH (≤1 µM) caused strong cytotoxicity. Interestingly, compared to the cells we used, flavonoids like quercetin exert a lower cytotoxic effect in the human hepatocellular cell line

(C.A. Musonda et al., 1998). On the other hand, the pre-incubation time with the flavonols in the experiments of K. Hyun-Jung was substantially longer and the chosen B[a]P concentration was lower than ours. Furthermore, J. Kinjo et al. (2006) showed that HepG2 cells were protected against the cytotoxicity exerted by *tert.*-butyl hydroperoxide (3h exposure) when the cells were pre-incubated for 1 hour with 35 µM quercetin or 72 µM kaempferol (EC_{50} values). These results emphasised that there are obvious tissue-specific differences in the cytotoxicity of flavonoids which are probably due to the particular molecular machinery handling the compounds in these cells. Therefore, the incubation conditions and analytical methods used to measure cytotoxicity have to be critical compared.

4.4. Kinetics of effects elicited by B[a]P, quercetin and kaempferol: Definition of suitable time points for our studies

We selected 4, 24 and 48 hours incubation time points for setting an effective concentration of B[a]P, quercetin and kaempferol in order to get an idea of the time-dependent effect of the individual compounds on the gene expression of the members of the AhR/Nrf2 pathway in the tested cell lines. We showed, for instance, that 10 µM B[a]P clearly induced the expression of CYP transcripts in CaCo-2 and IEC-6 cells after 4 hour exposure (fig. 3.4. and 3.13). A similar effect was observed by T. Walle et al. (2007) on CYP1A1 gen-expression in HepG2 after 6 hours incubation with 1 µM B[a]P. As already noted, the expression of most of the genes modulated by the AhR- or Nrf2-pathway are expressed at very low levels by HuTu-80 cells, which required more time (48h) for showing any effect exerted by the flavonoids and the carcinogen. For instance, only higher concentrations of B[a]P tended to induce CYP1B1 and AhR expression in these cells.

Quercetin (10 µM) clearly increased the expression of AhRR and CYP1A2 genes after 48 hours in CaCo-2 cells. Comparatively, H.P. Ciolino et al. (1999) observed a rapid increase in CYP1A1 transcript after 12h of treatment with 0.5 µM quercetin in MCF-7. Kaempferol rapidly (4h incubation) induced the expression of CYP in our experiments, reaching the strongest signal after 48 hours. Also the human duodenal cell line exhibited a maximal effect of both flavonoids after 48 hours incubation. IEC-6 treated cells showed induction of AhRR, CYP1A1 and AiP gene expression after 4 hours incubation whilst kaempferol induced the expression of several genes at its highest after 24h incubation.

In several studies it has been shown that PAH and flavonoids modulate either the mRNA, the protein level or the activity of cytochromes P450 (or all of these parameters), especially of CYP1A1. So far, these effects were observed in different cell lines (H.P. Ciolino et al., 1999; T. Walle et al., 2006; S. Plöttner et al., 2008), or at different time-points or the cells were incubated with different AhR-agonists or phytochemicals (C. Bonnesen et al., 2001; M.F. Yueh et al., 2002; C. Pohl et al., 2006). Because of this heterogeneity in method designs, it proved to be difficult to compare our results with other studies. We decided to stimulate both cell lines for 48 hours with B[a]P or flavonoids (10 µM each) since this concentration did exert a clear effect on the gene expression and higher concentrations of any of these substances did not show additional effects. This allowed us to compare the modulatory effects of the flavonols on both cell lines and, on the other hand, the 48 hours incubation allowed us to detect a clear protein expression of CYPs and a possible corresponding EROD activity as described by H.P. Ciolino et al. (1999), Y. Sakai et al. (2006) and S. Plöttner et al. (2008).

4.5. Modulatory effect of quercetin and kaempferol on the B[a]P-induced effects

Interestingly, in CaCo-2 and IEC-6 cells quercetin and kaempferol induced different mechanisms to modulate the B[a]P-induced gene expression. Attention should be paid to the fundamental varieties of the two cell lines derived from different tissues. For instance, B[a]P-induced a time-dependent reduction of AhRR expression in CaCo-2 (fig. 3.4.a) and a time-dependent induction in IEC-6 cells (fig. 3.12.a).

Our results proved that in CaCo-2 cells B[a]P activates the AhR and induced the expression of CYP as target genes. Since CYP1A1 transcription is induced after 48 hours we could also detect CYP1A1 active protein which is able to metabolise 7-ethoxyresorufin. Simultaneously, the amount of AhRR and ARNT mRNA was reduced and the amount of the AhR and Nrf2 transcript was increased. This pathway induction is very important to reach an effective detoxification. For example, CYP1A1 activates B[a]P to a reactive metabolite and ROS could be produced during an abortive catalytic cycle (R. Barouki et al., 2001). The increasing oxidative stress would cause a reduction of ARNT transcription or would increase a proteosomal degradation of ARNT, as described by H. Choi et al. (2008). Because of this, AhR could miss its dimerisation partner and the CYP induction would be reduced at later time points. This regulatory loop also explains the decline of EROD activity after 72 hour incubation in the publication of Y. Sakai et al. (2006).

Quercetin and kaempferol are ligands of the AhR (H.P. Ciolino et al. 1999) and in several studies it was shown that quercetin induced the CYP1A1 expression; however, kaempferol did not cause any effect on CYP1A1 transcription (H.P. Ciolino et al. 1999; C. Pohl et al. 2006).

Our experiments showed that quercetin increased the amount of CYP1A1, 1B1, 1A2 and AhRR transcripts after 48 hour incubation. On the other hand, S. Tanigawa et al. (2007) and P. Yao et al. (2007) observed that quercetin caused an effectively Nrf2/ARE-mediated activity and increased the transcription of phase II enzymes like NOQ1 or HO-1 (NAD(P)H dehydrogenase (quinone) 1 or Heme oxygenase 1). According to our results, however, in CaCo-2 cells the Nrf2-pathway does not seem to play an important role as the AhR-pathway. The ratio of the two transcription factors clarified the main focus of metabolism in this cell line. Interestingly, the AhRR is one of the lowest expressed members of the AhR-pathway. In our experiments quercetin caused an increase of the repressor transcript. This confirmed that the repressor is a target gene of the AhR transcription factor. On the other hand, the low expression level of AhRR does not affect the activated AhR-pathway. In line with our observations, Y. Tsuchiya et al. (2003) and K. Gradin et al. (1999) observed that only a high expression level of AhRR was really able to negatively regulate the AhR-pathway.

We observed that B[a]P and quercetin alone or in combination reduced the amount of ARNT transcripts significantly. We suggest that the metabolic CYP1A1 activity caused an increase of ROS which negatively regulated the expression of ARNT, the dimerisation partner of AhR. Comparatively, H. Choi et al. (2008) described that the polyphenol curcumin inhibited HIF-1α via ROS-dependent degradation of ARNT. On the other hand, G.N. Kim et al. (2009) showed that quercetin could act as an antioxidant in HepG2 cells when treated with quercetin for 30 min, but it acted as a pro-oxidant when the cells were incubated for longer periods (12 and 24 h). This result supports our hypothesis that quercetin is a ligand of AhR, induces the CYP expression and causes the synthesis of active CYP proteins (see western blots and EROD activity results). Quercetin is metabolised by CYP (T. Walle et al. 2004) and in this way ROS accumulate and inhibit the ARNT expression. Contrastingly, quercetin activated the HIF-1α- pathway in HepG2 cells by stabilising the HIF-1α protein. Since ARNT is also the heterodimeric partner of this gene regulatory protein the expression of ARNT seemed not to be reduced by this flavonoid. The results of J. Wilson et al. (2002) do not fit to our observation. Interestingly, C. Pohl et al. (2006) showed that CaCo-2 cells exposed to

quercetin showed a lower CYP1A1 mRNA induction than when exposed to TCDD and had no EROD activity. These results are in contrast to our observations and also to that of H.P. Ciolino et al. (1999).

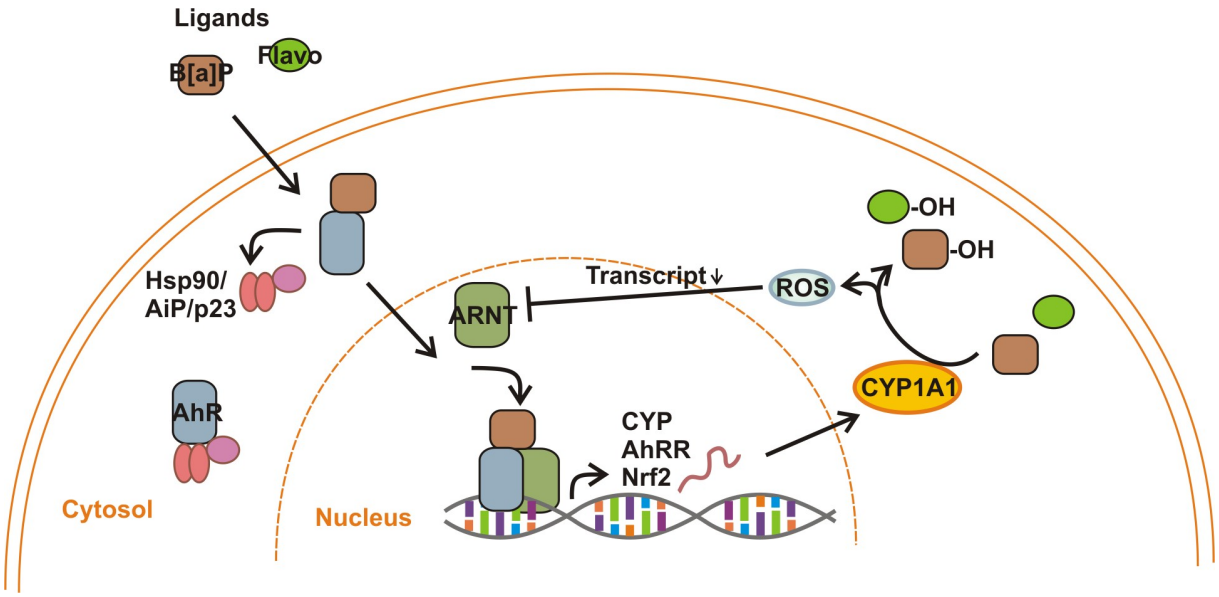


Fig. 4.1. Inhibition of ARNT transcription by increased ROS accumulation in CaCo-2 cells. The ligand binds the cytosolic AhR ligand-binding subunit, which undergoes a transformation or activation process involving several steps: translocation in the nucleus; release of a molecular chaperone complex containing at least Hsp90, AiP and p23, and dimerisation with a protein partner, ARNT. This heterodimer interacts with regulatory DNA sequences located upstream in the promoter of target genes and stimulate the transcription of genes in the CYP1A1. Quercetin is metabolised by CYP, simultaneously, ROS accumulates and inhibits the ARNT expression.

B[a]P and quercetin alone increased the expression of CYP1 especially that of CYP1A1 in human colon cell line. On the one hand we observed that in the combination experiments quercetin caused a slight additive stimulation on the expression of the CYPs (similar to D. Puppala et al., 2006). On the other hand, the B[a]P-mediated increased expression of Nrf2 and its target gene GCS tended to be antagonistically reduced. This confirms that the main modulating and effective target of flavonoids is the AhR-pathway in CaCo-2 cells. Surprisingly, the EROD activity was not proportionally increased to the induced amount of CYP protein. C. Pohl et al. (2006) illustrated that quercetin decreased the TCDD- induced EROD activity in CaCo-2 cells. This supports the idea that besides acting in transcriptional activation quercetin modulates the enzymatic properties of CYP (see fig. 3.9.b). However, other researchers showed that quercetin acted as an antagonist or did not affect the induction by TCDD (T. Walle et al., 2007; S.W. Allen et al., 2001).

In contrast, kaempferol alone influenced neither the gene nor the protein expression nor the EROD activity in our experiments. We did not identify this flavonoid as an agonist of the AhR. Interestingly, our results exhibit that this flavonol reduced the metabolic activity of CYP shown by our EROD assay results (fig. 3.8.b and 3.9.a). Kaempferol acted in a different way as quercetin, but it also inhibited the B[a]P-mediated activity of CYP1A1. On the other hand, there are a lot of evidences that kaempferol binds to the AhR, abolishes TCDD-induced XRE binding and decreases the TCDD-induced CYP1A1 transcription (H.P. Ciolino et al., 1999; R. Mukai et al., 2009). These differences in the modulatory activity of both flavonoids seemed to depend on the used cell line model and on the pre-incubation time.

Interestingly, the additive stimulation of the CYP1A1 induction offers evidence that quercetin acted as a ligand of the AhR in CaCo-2 cells, but it bound to a different binding site of the AhR as B[a]P did. Kaempferol could bind also on a different binding site of the AhR, but it did not modulate the B[a]P-activated AhR in colon derived cells. Both flavonoids reduced the CYP activity in a different dimension and may thus modulate the formation kinetics toxic metabolites and carcinogens in that way. F. Iori et al. (2005) showed that the amount of hydroxyl groups was important to get trapped into a polar pocket in proximity to the heme site of CYPs. Ligands presenting structures less crowded by hydroxyl substituents accommodate deeper into the binding site. Compared to quercetin, kaempferol has a similar polyphenolic structure but misses one hydroxyl group. Because of this, kaempferol is able to affect the enzymatic structure stronger and in a different way as quercetin does. The observation of F. Ioris et al. (2005) is in line with our results in the human colon cell line.

Also, in the rat cell line B[a]P caused a clear induction of the CYPs and the AhRR. The level of AhRR was as high as the level of the AhR after induction with B[a]P or flavonoids. We were able to detect very slight bands of the repressor protein by means of western blots, but the repressor protein did not reduce the high induction of CYP1A1 and the enormous expression of CYP1B1. Compared to the expression of CYP in CaCo-2 cells, IEC-6 showed a similar amount of induced CYP1A1 transcripts and a ~ 400 fold higher amount of CYP1B1 mRNA. Even with a strong transcriptional activity the amount of translated protein was still very low (see fig. 3.15) and the EROD activity was undetectable. A. Lampen et al. (1998) suggested that IEC-6 and HuTu-80 cells neither expressed CYP mRNA nor protein and he also could not detect any metabolic activity. On the other hand, he did not incubate the cells with PAH to induce CYP transcription. So, he analysed the cell lines for constitutively

expressed CYPs. It is known that human and rat CYP1A2 show only 75% homology that cause different amino acid structure (V.A. Eagling et al., 1998). Also, the size of the AhR-protein can differ dramatically between species and strains of mice (Y.Z. Gu et al., 2000). The AhR-dependent sensitivity to toxic substances also differs between varying species (K.W. Bock et al. 2006). This variability could explain our observed differences in gene and protein pattern of the human and rat derived cell line. This possible species- or/and tissue dependent strategy of detoxification, elimination or avoiding toxification of xenobiotica also shows different evolutionary adaptation to their environment. P.H. Roos et al. (2004) observed that the PAH-mediated induction of CYP1A1 differs in a tissue-specific and dose-dependent manner in duodenum, liver and kidney of minipigs and rats. The rapid detoxification and elimination of xenobiotics can avoid an accumulation of potential carcinogens in the cell, DNA damage and cancer development. Adaptive mechanism like increasing the transcription of an enzyme has to occur in the right time. Our results pointed out that both tested substances caused induction of gene expression at earlier time-points. This could explain why IEC-6 showed a different gene and protein pattern induced by B[a]P, quercetin or kaempferol after 48 hours incubation. Further investigations could give information about early degradation of proteins or time-depended inhibition of translation in the rat duodenal cell line.

Besides the receptor level, the induced AhR activity also depends on phosphorylation of the receptor by the protein kinase C (PKC) (Y.H. Chen et al., 1996; Y.C. Cho et al., 2004; L. Cocco et al., 1992). It is also described that the accumulation and the activity of Nrf2 depends on phosphorylation, too. The PKC has been shown to interfere with Nrf2 binding to Keap1 (T. Nguyen et al., 2003) which supports an enhanced Nrf2-acitivty. Noticeably, the Nrf2-pathway is a major pathway modulated by B[a]P in duodenal cell lines especially in the IEC-6. Our results showed that members of the Nrf2-pathway dominated over other members of the AhR pathway after 48 hours incubation (table 3.5.) and this could lead to a faster elimination of reactive metabolites. X.L. Tan et al. (2009) suggested in his review that Nrf2 was responsible for phase II gene expression but not for phase I induction.

In order to dissect the gene expression pattern exerted by flavonols on the B[a]P-induced effects we exposed both cell lines to the selective Nrf2 activator ethoxyquin (EQ) and the AhR/Nrf2 mixed activator *tert*-butylhydroquinone (tBHQ) as described by C. Köhle et al. (2006) and W. Miao et al. (2004). However, there is evidence that AhR- and Nrf2-pathways are coupled (C. Köhle et al., 2007 and S. Sin et al., 2007) meaning that Nrf2 is a target gene

of the AhR-pathway and/or the other way around. By activating these coupled pathways, we expected that either EQ or tBHQ should induce the expression of the target genes (excepting that of ARNT). If in these intestine cell lines Nrf2 is not coupled with the AhR-pathway, then EQ would only increase the expression of GCS. In our experiments EQ strongly increased only the CYP1A1 transcript whilst the AhR mRNA was not induced. On the other hand, the mix-activator tBHQ caused a similar gene expression pattern in the cell lines. We can point out that these substances caused similar inductive effects which differed from those exerted by B[a]P, quercetin or kaempferol. Since tBHQ is known as a ligand of AhR it is not remarkable that it induced the CYP expression (W. Miao et al., 2004 and C. Köhle et al., 2006). In contrast, EQ first has to be activated by CYPs to an electrophilic metabolite to act as a specific Nrf2 ligand. This means also that CYP expression has to be induced. T.M. Buetler et al. (1995) showed that EQ actually increased CYP transcription. These results agree with our observations.

Furthermore, phosphatidylinositol 3-kinase (PI3K) also has been identified to be essential for the nuclear translocation of Nrf2 and for Nrf2 DNA binding. PI3K also phosphorylates the CCAAT/enhancer binding protein-β (C/EBP- β), inducing its translocation to the nucleus and binding to the CCAAT sequence of C/EBP- β response element with XRE, in conjunction with Nrf2 binding to ARE (X.L. Tan et al., 2009; I.J. Cho et al., 2003). That finding also supports the idea of an AhR-independent CYP1B1 induction in the IEC-6 cells. Furthermore, G. Agullo et al. (1997) reported about a quercetin and kaempferol-dependent inhibition of PI3K activity. It explains our results with the flavonoids in this cell line. In agreement with our hypothesis and our observations, B[a]P also induces the PI3K activity (S.L. Tannheimer et al., 1998). That means that B[a]P could cause an AhR-independent CYP expression even with an inhibited AhR-pathway by AhRR.

After 48 hours of B[a]P exposure, we could not detect any EROD activity in IEC-6 cells although the expression of CYP1B1 reached a high level. This result indicates that there could be a lack of stabilisation factors of the CYP1B1 mRNA, a fast degradation of the transcripts, an inhibition of the translation or a fast inactivation and degradation of the CYP protein that could explain our observation. Further investigations are needed to understand the regulatory mechanism in the IEC-6 cell line. Z.C. Kang et al. (1999) and M.Y. Heo et al. (2001) reported that quercetin inhibits the protein kinase C resulting in inhibition of the AhR activity. It explains our observation that neither quercetin nor kaempferol strongly induced the CYP

expression or other members of the pathways in the IEC-6 cells. On the other hand, quercetin and kaempferol are known inhibitors of protein kinase A (PKA), but the same researchers also observed that both flavonoids induced the cAMP-dependent PKA (W.S. da-Silva et al. 2007 and J.T. Sanderson et al. 2004). E. Eliasson et al. (1992) showed that a PKA-dependent phosphorylation of CYP2E1 leads to its degradation. It is not know that this is also the case for CYP1-enzymes but it could be the cause in the IEC-6 cell line (B. Oesch-Bartlomowiczet al., 2002). This could also explain the missed EROD activity.

Another possibility is the inhibition of translation by the PI3K/Akt-dependent activation of the mTOR-pathway. The mTORC1 complex (composed of mTOR, Raptor, mLST8/GβL and PRAS40) plays a role in the regulation of translation by means of activation of two key proteins, 4E-BP1 and S6K1. 4E-BP1 interacts with eIF4E and inhibits recruiting 40S ribosomal subunits to the 5' end of mRNAs and represses translation. Active S6K1 can in turn stimulate the initiation of protein synthesis through activation of S6 Ribosomal protein (a component of the ribosome) and other components of the translational machinery. The activated mTORC1 complex phosphorylates this key proteins what results in the initiation of translation. This mTOC1-mediated activation of protein translation is inhibited by suppressor protein TSC1/TSC2. The PI3K-activited Akt/PKB phosphorylates the TSC1/TSC2 and activates indirectly the mTORC1 which causes an induction of protein synthesis. Quercetin and kaempferol are able to inhibit the PI3K (as already noted above) and caused an inhibition of protein synthesis. Microarray analysis provides an insight into the expression of many genes in the untreated and treated IEC-6 cells. In contrast to our hypothesis, our microarray results offer no clear evidence for a possible mechanism in this cell line which leads to the inhibition of CYP1B1 and CYP1A1 translation to avoid an accumulation of reactive metabolites (fig. 4.2.). For example, the PTEN-mediated dephosphorylation could inhibit Akt-mediated mTORC1 activation in IEC-6. This hypothesis has to be examined experimentally by means of western blots analysis and gene silencing using siRNA in combination with CYP protein and activity analysis (EROD).

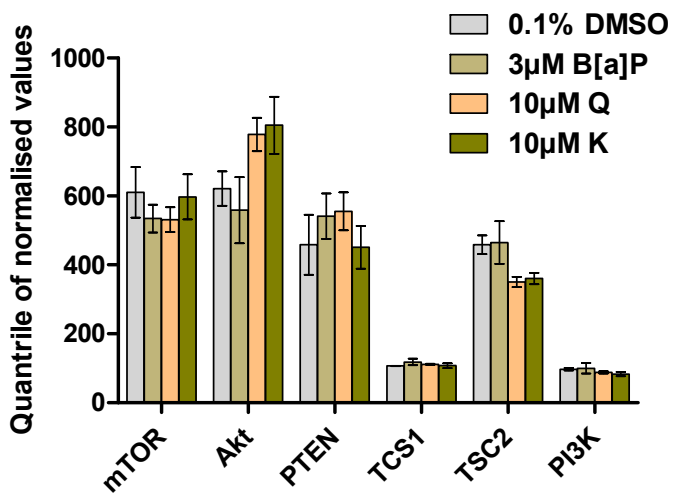


Fig. 4.2. Result of microarray analysis of untreated and treated IEC-6 cells. Cells were incubated with B[a]P, Q or K for 48h. The isolated total RNA was analysed be means of microarray assay. The mean value ± standard deviations of three independent experiments are depicted.

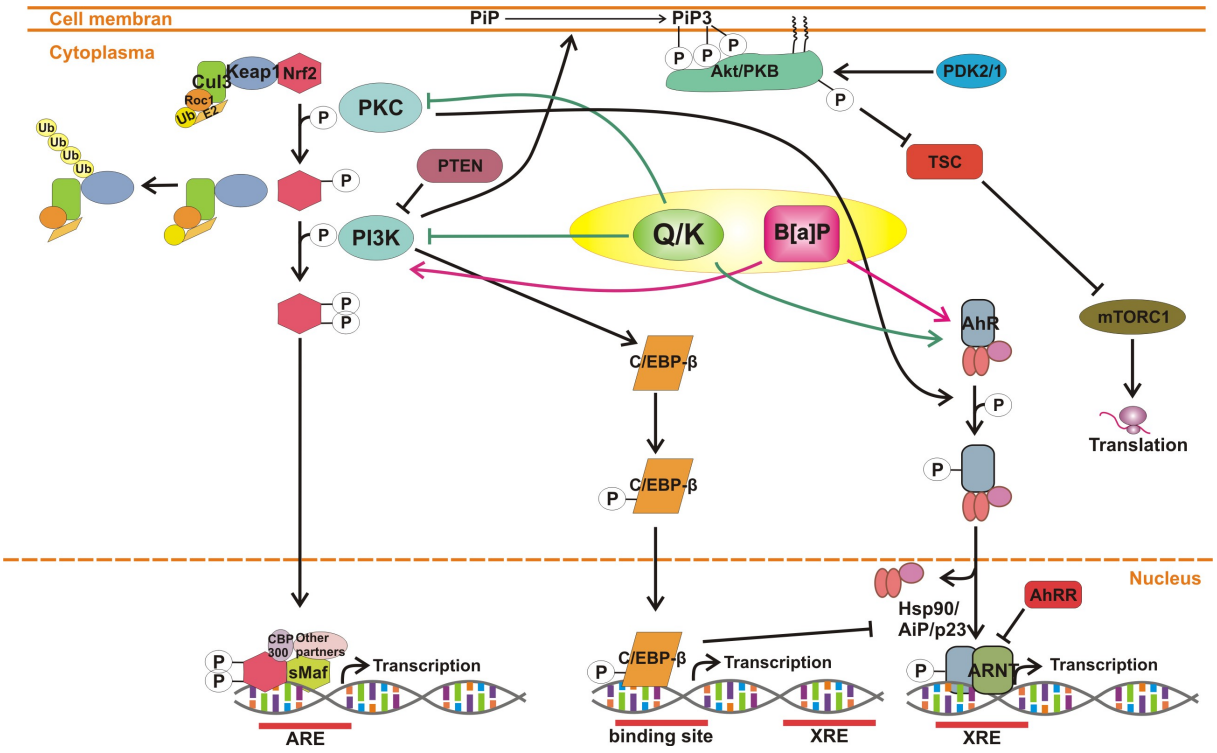


Fig. 4.3. Overview of the cross-talking pathways in the IEC-6 cells. PKC and PI3K are important for the activation of the Nrf2-pathway. The phosphorylation of Nrf2 by the PKC is necessary for Nrf2 release from Keap1. The phosphorylation by PI3K has been identified to be essential for the nuclear translocation of Nrf2 and the binding of the Nrf2-complex to DNA. PI3K is also a key protein in the regulation of the mTORC1-mediated protein synthesis and the C/EBP-β-mediated cytochrome P450 1A1 and 1B1 transcription. Also, the phosphorylation of the AhR by PKC is essential for the dimerisation with ARNT and DNA binding. The flavonoids Q and K and also B[a]P are interacting with the transcription factors and protein kinases.

4.6. Cell line models in comparison to the *in vivo* situation

The human duodenal cell line, HuTu-80, exhibits low gene expression levels concerning AhR- and Nrf2-pathway components in comparison to IEC-6 and CaCo-2 cells. Especially, the gene expression level of AhR, AhRR and CYPs (member of the AhR-pathway) is very low in this cell line. Noticeably, the expression of Nrf2, ARNT and GCS is approximately 10- to 100-times higher than that of the AhR-pathway members. Even after incubation with B[a]P, quercetin or kaempferol we could not detect any induced CYP expression. Similar to the observations in the IEC-6 cells, we observed a tendency of enhanced expression of Nrf2 and GCS after treatment with the chemicals. To our knowledge, we are the first who investigate the relevance of the coupled AhR- and Nrf2-pathway in human duodenal cell lines. J.S. Petrick et al. (2007) evidenced that the AhR-pathway plays a secondary role in the elimination of xenobiotics in the murine duodenum. It seemed that the Nrf2- followed by the PXR-pathway pre-dominates the signal transduction in the mouse duodenum. Also M.K. Shelby et al. (2006) described that the Nrf2-pathway in rat duodenum predominates over the AhR-pathway. Our results in the IEC-6 cells agree with these observations. Also, in human duodenal tissue it was demonstrated that the AhR-pathway seemed to be a secondary pathway (Q.Y. Zang et al., 1999; D. Sun et al., 2002; L.S. Kaminsky et al., 2003). By contrast, oral intake of particle-bound PAH led to an increase of the enzymatic capacity through induction of CYP1A1 in the duodenal cells of minipigs (P.H. Roos et al., 2004). N. Hernández-Matínez et al. (2007) and D. Mitschke et al. (2008) detected a slight constitutive CYP1A1 protein expression in rat duodenum. After treatment with phenobarbital/naphtoflavone the CYP1A1 protein expression was enhanced and also the EROD activity became noticeable. These observations were made in wistar and wistar-HAN rats. We have no information about the rat stem origin of the IEC-6 cell line. For example, S. Yasuo et al. (2007) recognised that there are differences in gene expression profiles and physiology between different rat stems. This could be an explanation for the difference in cellular regulation. Nevertheless, we should keep in mind that isolated and immortalised cells cannot behave like a whole organism. Furthermore, studying the regulatory mechanism in IEC-6 cells seems to provide important information about cellular protection mechanism against possible carcinogens.

Gene expression profiles obtained by means of microarray analysis showed that CYP2C9, 2C18 and 3A4 are the highest expressed CYP genes in human duodenum (D. Sun et al., 2002). However, the CaCo-2 cell line showed a different CYP expression profile and

CYP3A4 was much lower compared to that in the human tissue (D. Sun et al., 2002; Q.Y. Zang et al., 1999). On the contrary, the AhR-pathway seemed to play an important role in the metabolism and elimination of xenobiotics in CaCo-2 cells. Induced CYP1A1 protein was detected in human small intestine biopsy samples (J. Buchthal et al., 1995; M.F. Paine et al. 1999). However, the biopsy results often derived from jejunum and they misrepresent the real situation in the human duodenum. Since the distribution of CYP showed a specific expression profile along the intestinal segments of the rat and also a remarkable inter-individual variability (D. Mitschke et al., 2008) we can not really exclude the HuTu-80 cell line as a model for human duodenal cells. Further, there is enough evidence that CaCo-2 cells are a good model for studies on the regulation of the AhR-pathway. However, CaCo-2 cells can not be used as a human duodenal cell line model.

It was shown by several groups that CYP1A1 expression is inducible in human duodenum (J. Buchthal et al., 1994 ; M.F. Paine et al., 1998; T.N. Johnson et al., 2008) . These observations are in contrast to our results obtained with the human duodenal cell line. Interestingly, both duodenal derived cell lines studied exhibit a similar regulation profile. Further investigations about the PXR-pathway and protein expression analysis of member of the Nrf2-pathway in the HuTu-80 and IEC-6 cells are necessary to prove our hypothesis that for example phosphorylation via PKC and PI3K could play a role in the regulation of gene expression or a specific regulation of the mTOR-Complex which modulates the protein synthesis and to understand the molecular mechanism of cell protection by flavonoids.

5. Summary

Benzo[a]pyrene (B[a]P), a polycyclic aromatic hydrocarbon, is considered a widespread pollutant and carcinogen. It acts as a ligand of the AhR and induces the gene expression of CYP1A1. This induction of CYP1A1 is an initial step in chemical-induced carcinogenesis. Flavonoids like quercetin and kaempferol which are present in human diet and absorbed in the small intestine can modulate this transcriptional and enzymatic activation. Since an efficient and rapid elimination of toxic substances depends on the coupling of phase I and II enzymes, the balance of their activities determines the extent of adverse effects with degenerative diseases and cancer as endpoints, for example. We studied, at the transcriptional, protein and enzymatic activity level, the modulatory effect of flavonoids on the B[a]P-induced expression of components and target genes of the AhR- and Nrf2-pathway in human and rat duodenum (HuTu-80 and IEC-6, respectively) and human colon (CaCo-2) derived cell lines. Noticeably, the phase I metabolism induced via the AhR-pathway seemed to play an important role in CaCo-2 cell line compared to HuTu-80 or IEC-6 cells. B[a]P and quercetin induced the CYP1A1 gene expression and protein levels as well its enzymatic activity. However, kaempferol did not affect any member of AhR- and Nrf2- pathways by itself. Interestingly, both flavonoids inhibited the B[a]P-induced CYP activity. This points out that the phytochemicals were able to modulate the kinetics of reactive metabolite formation and of carcinogen activation. Simultaneously, the ARNT transcription was even stronger reduced by B[a]P plus each flavonoid which is probably due to CYP-mediated ROS production. This feedback mechanism would also result diminished formation of reactive metabolites. In contrast to the CaCo-2 cells, the phase II metabolism via Nrf2-pathway seemed to play an important role in rat and human duodenal cell lines. In spite of the high B[a]P-mediated induction of CYP1B1 and CYP1A1 gene expression, slight protein expression and no EROD activity were detected in IEC-6 cells. Also in combination with each flavonoid, the CYP gene and protein expression was unaffected. It is concluded that the absence of CYP1 protein results in limited capability to generate toxic/reactive metabolites. On the other hand, the Nrf2 and GCS gene expression was increased by B[a]P and enhanced in combination with quercetin or kaempferol in IEC-6 cells. Furthermore, the high expression level of the Nrf2-pathway compounds suggested a fast elimination of xenobiotics via induced phase II activities. However, HuTu-80 cells generally exhibited very low gene expression of the studied receptor pathway components and enzymes. This fact prohibited us to draw any clear conclusions about the effects of B[a]P, quercetin and kaempferol in human duodenal cells.

6. Zusammenfassung

B[a]P, ein polyzyklischer aromatischer Kohlenwasserstoff, ist ein in der Umwelt weit verbreitetes Karzinogen. Es fungiert als Ligand des AhR und induziert dadurch z.B. die CYP1A1 Genexpression. Diese Induktion ist der Ausgangspunkt für die chemisch induzierte Karzinogenese. Die in pflanzlicher Nahrung vorkommenden Flavonoide wie Quercetin und Kaempferol hingegen können diese transkriptionelle und die resultierende enzymatische Aktivität modulieren. Da eine effiziente und schnelle Elimination von toxischen Substanzen von der Verknüpfung des Phase I und II Stoffwechsels abhängt, stellt sie eine Art Schlüsselmechanismus hinsichtlich des Ausmaßes adverser Effekte dar. Daher kann sie als Ansatzpunkt für Präventivmaßnahmen gegen degenerative Erkrankungen oder die Krebsentstehung genutzt werden.

Wir haben auf Transkript-, Protein- und Enzymaktivitätsebene die Modulationsfähigkeit von Flavonoiden auf die B[a]P induzierte Expression von Komponenten und Zielgenen des AhR- und Nrf2-Signaltransduktionsweges in verschiedenen Darmzelllinien untersucht: HuTu-80 und IEC-6 aus dem Duodenum von Menschen bzw. Ratte sowie CaCo-2 aus dem menschlichem Kolon. Die Aktivierung des Phase I Metabolismus über den AhR-Siganltransduktionsweg ist in den Kolonzellen (CaCo-2) wesentlich höher als in den beiden Duodenumzelllinie (HuTu-80 und IEC-6). Sowohl B[a]P als auch Quercetin induzierten die CYP1A1 Gen- und Proteinexpression sowie auch dessen Enzymaktivität. Kaempferol alleine hingegen nahm keinen Einfluss auf die Expression von Komponenten der AhR- und Nrf2-Signaltransduktionswege. Beide Flavonoide vermindern die B[a]P induzierte EROD-Aktivität. Dies zeigt, dass sekundärer Pflanzenstoffe die Kinetik zur Bildung von toxischen Metaboliten und der Karzinogen-Aktivierung modulieren können. Gleichzeitig war die Transkription von ARNT durch die Kombination aus B[a]P und Flavonoid noch stärker reduziert, welche vermutlich aus der CYP bedingte ROS Produktion resultierte. Diese Rückkopplung kann somit zusätzlich zu einer verminderten Bildung reaktiver Metabolite führen. Im Gegensatz zu den CaCo-2 Zellen, zeigten die Duodenumzelllinien eine stärkere Aktivierung des über den Nrf2-Siganltransduktionsweg induzierten Phase II Metabolismus, dem in diesen Zellen offenbar eine wichtigere Rolle zukommt. Trotz der starken Induktion der CYP1B1 und CYP1A1 Genexpression waren nur geringe Mengen von CYP1A1- Protein und zudem keine EROD-Aktivität in den IEC-6 Zellen zu detektieren. Beide Flavonoide zeigten in den IEC6-Zellen keinen Effekt auf die CYP-Induktion durch B[a]P. Das Fehlen von CYP1 Proteinen hat vermutlich eine reduzierte Produktion toxischer Metaboliten zur

Folge. Andererseits wurde die Nrf2 und GCS Genexpression durch B[a]P erhöht und in Kombination mit Quercetin oder Kaempferol noch weiter verstärkt. Darüber hinaus deutet der hohe Expressionslevel der am Nrf2-Siganltransduktionsweges beteiligten Komponenten auf eine schnellere Elimination von Xenobiotika hin. Die HuTu-80 Zellen weisen generell einen geringen Genexpressionslevel der untersuchten Komponenten auf, der es uns nicht möglich macht klare Schlussfolgerungen über die Wirkungen von B[a]P, Quercetin und Kaempferol in humanen Duodenalzellen zu ziehen.

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9. Curriculum Vitae

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I prepared the present dissertation independently and without any impermissible help. The thesis has not been submitted in the present form to any other institution. Hitherto, I have not performed unsuccessful efforts to get a doctoral degree.

Jeanette Luise Niestroy

Dortmund, November 11th 2009