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The impact of the phenolic derivatives eugenol and isoeugenol on the aryl hydrocarbon receptor signalling pathway in human keratinocytes (HaCaT)

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II Abbreviations

%	percent
µ	micro
AhR	aryl hydrocarbon receptor
AhRR	aryl hydrocarbon receptor repressor
AIP	AhR interacting protein
ARA9	AhR associated protein 9
ARNT	aryl hydrocarbon receptor nuclear translocator
B[a]P	benzo[a]pyrene
bHLH	basic helix loop helix
bp	base pairs
BrdU	5‘-bromo-2‘-deoxyuridine
BSA	bovine serum albumin
CDK	cyclin dependent kinase
cDNA	complementary DNA
cf.	confer
CKI	cyclin dependent kinase inhibitor
COX-2	cyclooxygenase-2
CYP450	cytochrome P450
DMBA	7,12-dimethylbenz[a]antracene
DMEM	Dulbecco´s modified eagle medium
DMSO	dimethyl sulfoxide (Me_2SO)
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleosidtriphosphate
DRE	dioxin responsive element
E2F	E2 factor
EDTA	ethylenediaminetetraacetic acid
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
FCS	fetal calf serum
g	gravitation or gram
G_0 phase	gap_0 phase
G_1 phase	gap_1 phase
G_2 phase	gap_2 phase
h	hours
HaCaT	spontaneously immortalized human keratinocyte cell line Ha=human adult, Ca=calcium, T=temperature

HAH	halogenated aromatic hydrocarbon
HSP 90	heat shock protein 90
IF	immunofluorescence
IgG	Immunoglobulin G
kDa	kilo Dalton
LC	Light Cycler
mA	milliampere
min	minute
ml	millilitre
mM	millimolar
MNF	3´-methoxy-4´-nitroflavone
M-phase	mitosis-phase
mRNA	messenger RNA
NES	nuclear export signal
NLS	nuclear localization signal
nm	nanometer
PAH	polycyclic aromatic hydrocarbon
PAS	Per-Arnt-Sim
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pg	picogram
PI	propidium iodide
pRB	retinoblastoma protein
RNA	ribonucleic acid
RNAse	ribonuclease
RT	reverse transcription
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
siRNA	short interfering RNA
S-phase	synthesis-phase
t	temperature
TAE buffer	tris-acetate buffer
Taq	*Thermus aquaticus*
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TGF-ß	transforming growth factor beta
Tm	melting temperature
TNFα	tumour necrosis factor alpha

U	units
V	Volt
WST	4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate
XRE	xenobiotic response element

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1. Introduction

The skin is the largest organ of the human body. As an interface between an individual and its environment it is a major site of direct exposure to many naturally occurring as well as xenobiotic substances. The frequent application of creams or natural and synthetic body care products is a major source of exposure, among others. Such products contain ingredients like alcohols, lipids, dyes, vitamins, and fragrances. Once absorbed, these substances may interact with different biomolecules in the skin and specifically or non-specifically induce signal transduction pathways. A variety of physiological mechanisms in skin has been evolved to adapt to both, endogenous stimuli and exogenous insults. These events may result in various physiological responses including homeostatic perturbations, cellular responses affecting proliferation, differentiation, apoptosis, or necrosis, among others, but also in a number of diseases and pathophysiological outcomes.

The aryl hydrocarbon receptor (AhR) signalling pathway is one mechanism whereby the skin responds to xenobiotic and naturally occurring substances, predominantly through the induction or upregulation of metabolizing enzymes. One known physiological function of the AhR in many tissues is its involvement in the control of cell cycle progression. In contrast to xenobiotic metabolism, almost nothing is known about this endogenous function of the AhR in skin. Moreover, the question whether naturally occurring substances like fragrances impact on the AhR signalling pathway within the skin has rarely been studied so far and is the focal point of this thesis.

1.1 Eugenol and isoeugenol

Phenolic derivatives like eugenol (4-allyl-2-methoxyphenol) and its isoform isoeugenol (2-methoxy-4-propeylphenol) (figure 1) are naturally found in essential oils of different spices such as *Syzgium aromaticum* (clove), *Pimenta racemosa* (bay leaves), and *Cinnamomum verum* (cinnamon leaf). Due to their odour, eugenol and isoeugenol are referred to as fragrances. These two fragrances are ubiquitous in our daily lives and are not only present in cosmetic products but also in household products, medications, food, paper, and paints (Merk et al., 2007). Their ubiquitous distribution leads to an unavoidable exposure to skin and consequently, sensitization rates to eugenol and isoeugenol are very high in most countries (Buckley et al., 2000).

Apart from being a flavouring agent in cosmetic and food products, eugenol and isoeugenol exhibit antiseptic, antibacterial, and analgesic properties and therefore are used in traditional medicine (Ghosh et al., 2005). Eugenol possesses antiviral activity against human herpesvirus (Benencia and Courreges, 2000) and moreover, both eugenol and

isoeugenol are used in dentistry as ingredients in cavity filling cement (Ghosh et al., 2005). Although eugenol and isoeugenol are naturally occurring and toxicological harmless, several physiological activities of them have been described pointing to a cytoprotective role for both phenolic derivatives. Due to their antioxidative capacities and radical scavenger activities, eugenol and isoeugenol influence redox homeostasis of cells (Taira et al., 1992; Rajakumar and Rao, 1993; Fujisawa et al., 2002; Kadoma et al., 2007). Furthermore, eugenol and isoeugenol have anti-inflammatory properties for example by impacting pro-inflammatory mediators like prostaglandins (Rasheed et al., 1984; Huss et al., 2002). Moreover, eugenol is able to modulate metabolizing enzymes like glutathione S-transferases (Rompelberg et al., 1996) and cytochrome P450 1A1 (CYP1A1) (Al Masaoudi et al., 2001). *CYP1A1* is one of the best characterized target genes of the AhR and hence, it might be possible that eugenol directly or indirectly activates the AhR, thereby inducing *CYP1A1* mRNA. More recently, antiproliferative effects of eugenol were detected, supporting the hypothesis of its potential interaction with the AhR due to its impact on the control of cell cycle progression. Eugenol was found to cause melanoma growth suppression in mice, through the inhibition of E2 factor 1 (E2F1) transcriptional activity (Ghosh et al., 2005). In melanoma cell lines (Ghosh et al., 2005; Pisano et al., 2007) and oral KB carcinoma cells (Chang et al., 2002), eugenol causes growth suppression and moreover, apoptosis in melanoma cell lines (Ghosh et al., 2005; Pisano et al., 2007), mast cells (Park et al., 2005) and HL-60 leukemia cells (Okada et al., 2005). Furthermore, eugenol was reported as being partially effective in inhibiting benzo[a]pyrene-induced skin carcinomas (Van Duuren and Goldschmidt, 1976) also pointing to a putative interaction of eugenol with the AhR.

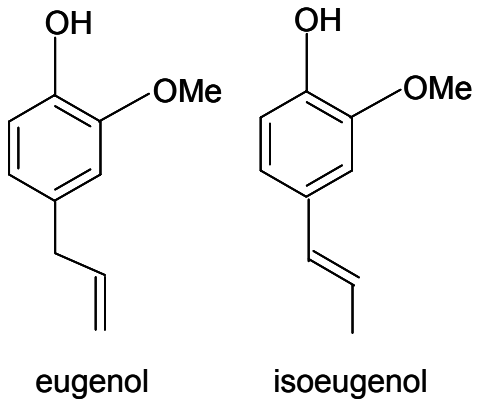


Figure 1: Eugenol and isoeugenol

Chemical structure of eugenol (4-allyl-2-methoxyphenol) and its isoform isoeugenol (2-methoxy-4-propenylphenol).

1.2 The Aryl Hydrocarbon Receptor

The field of AhR research has its origin in mammalian toxicology. The AhR was identified by Poland and colleagues in the 1970s (Poland et al., 1976) and it is now clear that the physiological response to many environmental substances is a direct consequence of their interaction with the AhR. An established role for the AhR in metabolism and thereby in toxicity of such substances demonstrates how certain classes of chemicals exert their toxicity and how mammalian organisms adapt to such exposures. Nevertheless, a phylogenetic survey indicates that the AhR arose over 450 million years ago, thus long before environmental pollution by anthropogenic compounds started (Hahn, 2002). A unique role of the AhR in defense against environmental chemicals is therefore very improbable. In recent years, interest in AhR biology has grown beyond a toxicological perspective, as research elucidated a physiological role for this receptor.

1.2.1 Domain structure of the Aryl Hydrocarbon Receptor

The AhR belongs to the family of basic helix-loop-helix (bHLH)- and Per-Arnt-Sim (PAS)-containing transcription factors (Hankinson, 1995; Ma, 2001). The bHLH motive is located near the N-terminus of these proteins (figure 2). DNA binding of the receptor occurs principally through the basic domains of these proteins, whereas association with other proteins involves their HLH domains. A nuclear localization signal (NLS) and a nuclear export signal (NES) is also found in the amino-terminal part of these proteins. The most characteristic region in sequence homology found in this family of proteins is termed the PAS domain, which was originally recognized because of the homology of the region in period (PER, a *Drosophila melanogaster* circadian protein), aryl hydrocarbon receptor nuclear translocator (ARNT, human protein), and single-minded protein (SIM, a *Drosophila melanogaster* protein involved in CNS development). The PAS domain consists of 250-300 amino acids and contains two copies of an approximately 50 amino acid degenerated direct repeat, referred to as the PAS A and PAS B repeats (Swanson and Bradfield, 1993). The AhR is unique among this protein family in that its activity depends on the binding of a ligand. Ligand binding to AhR occurs over the PAS B domain. The carboxy-terminal, glutamine-(Q)-rich segments of the AhR contain the transcriptional activation domain with a number of independently active subdomains. In contrast to the highly conserved bHLH- and PAS-domains, the carboxy-terminal segments of this protein family are variable (Hankinson, 2005).

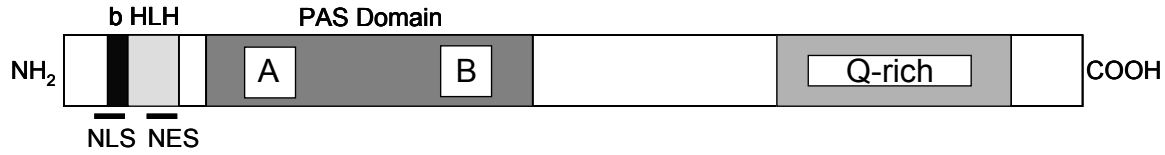


Figure 2: Domain structure of the aryl hydrocarbon receptor

NH_2: amino-terminus, bHLH: basic helix-loop-helix, PAS: Per-Arnt-Sim, A: PAS A, B: PAS B, Q-rich: glutamine-rich, COOH: carboxy-terminus, NLS: nuclear localization signal, NES: nuclear export signal. Adapted from Denison et al.,2002 (Denison et al., 2002).

1.2.2 Aryl Hydrocarbon Receptor signalling pathway

Basic helix-loop-helix proteins like the AhR are involved in a variety of physiological processes including the regulation of myogenesis (Neuhold and Wold, 1993) and neurogenesis (Cabrera and Alonso, 1991), the regulation of immunoglobulin genes (Zhao et al., 1993), cell proliferation (Amati et al., 1993; Gu et al., 1993), and xenobiotic metabolism (Swanson and Bradfield, 1993). For the AhR, xenobiotic metabolism is the best characterized physiological function.

In its unliganded form, the AhR is present in the cytosolic compartment as a multiprotein complex containing two polypeptides of the 90 kDa heat shock protein (HSP90) (Meyer et al., 1998), one polypeptide of the HSP90 associated co-chaperone p23 (Kazlauskas et al., 1999), and the immunophilin homolog XAP2 (Meyer et al., 1998; Pollenz et al., 2006), also called AhR interacting protein (AIP) (Ma and Whitlock, 1997) or AhR associated protein 9 (ARA9) (Carver and Bradfield, 1997) (cf. figure 3, (1)). Upon ligand binding (cf. figure 3, (2)), the entire cytosolic AhR complex is presumed to undergo a conformation change resulting in exposure of its nuclear localization sequence (NLS) and hence translocates into the nucleus (Ikuta et al., 1998). Once there, the AhR dissociates from the HSP90/p23/XAP2 complex (cf. figure 3, (3)) and simultaneously heterodimerizes with a second basic helix-loop-helix/PAS protein, the aryl hydrocarbon receptor nuclear translocator (ARNT) (Hankinson, 1995). Association of AhR/ARNT converts the complex into its high affinity DNA binding form whereupon it binds to its specific DNA binding sites, the xenobiotic responsive elements (XREs), also called dioxin responsive elements (DREs) (cf. figure 3, (4)) (Swanson et al., 1995), in order to regulate the transcription of several target genes (cf. figure 3, (5)) (Mimura and Fujii-Kuriyama, 2003).

AhR target genes can be divided into two main groups. The first group includes genes coding for phase I metabolizing enzymes belonging to the cytochrome P450 1 (CYP1) family (CYP1A1, CYP1A2, and CYP1B1) (Nebert et al., 2000) and phase II enzymes like

UDP glucuronosyltransferases (UGT1A1, UGT1A6) (Nebert et al., 2000; Yueh et al., 2003), glutathione transferase (GSTA1,Ya), NAD(P)H:quinone oxidoreductase (NQO1), and aldehyde dehydrogenase 3 (ALDH3A1) (Nebert et al., 2000). In addition, the transcription factor NF-E2p45-related factor (NRF-2) (Miao et al., 2005) and the AhR repressor (AhRR) (Mimura et al., 1999) also belong to that group, among others. The second group includes genes involved in proliferation, differentiation, and regulation of development. Among them are the genes coding for the cyclin dependent kinase inhibitors $p21^{CIP}$ (Barnes-Ellerbe et al., 2004) and $p27^{KIP}$ (Kolluri et al., 1999; Jin et al., 2004; Levine-Fridman et al., 2004), the immediate early proteins C-JUN and JUN-D (Hoffer et al., 1996), the bHLH transcription factor repressor HES-1 (Thomsen et al., 2004), the cytokine for homeostasis of T cells IL-2 (Jeon and Esser, 2000), the proapoptotic BCL-2 associated x protein (BAX) (Matikainen et al., 2001), the insulin-like growth factor binding protein (IGFBP-1) (Marchand et al., 2005), DNA-Polymerase κ (Ogi et al., 2001), and filaggrin, a key protein involved in skin differentiation (Loertscher et al., 2002). Numerous of other genes have been implicated as being modulated either directly or indirectly by the AhR. In many cases, the underlying mechanism remains to be studied because not all of them contain putative XRE elements in their promoters (Tijet et al., 2006).

The AhRR functions as a negative regulator of the AhR by competing with AhR for ARNT binding (cf. figure 3, (6)). Moreover, the AhRR/ARNT complex represses XREs (cf. figure 3, (7)). Upon gene transactivation, the AhR is exported from the nucleus to the cytoplasm and subsequently ubiquinated and degraded by the 26S proteasome pathway (Pollenz, 2002). This attenuation of AhR activity by means of a negative feedback loop and receptor degradation may serve to protect the organism from the consequences of transcriptional hyperstimulation by potent agonists and to provide precise temporal control of this pathway. In addition, multiple mechanisms of AhR signalling have been identified. Recently, a large number of coregulators have been detected which are involved in the interaction of AhR between enhancers and promoters (Hankinson, 2005). Furthermore, a direct interaction of the AhR with the retinoblastoma protein (pRB) and nuclear factor κB (NF-κB) has been identified. Moreover, AhR signalling includes cross-talk with a number of protein kinases, and evidence for ligand-independent activation of the AhR was obtained (Puga et al., 2002). The exact underlying molecular mechanisms of the physiological signalling pathway in which the AhR is involved remain to be studied.

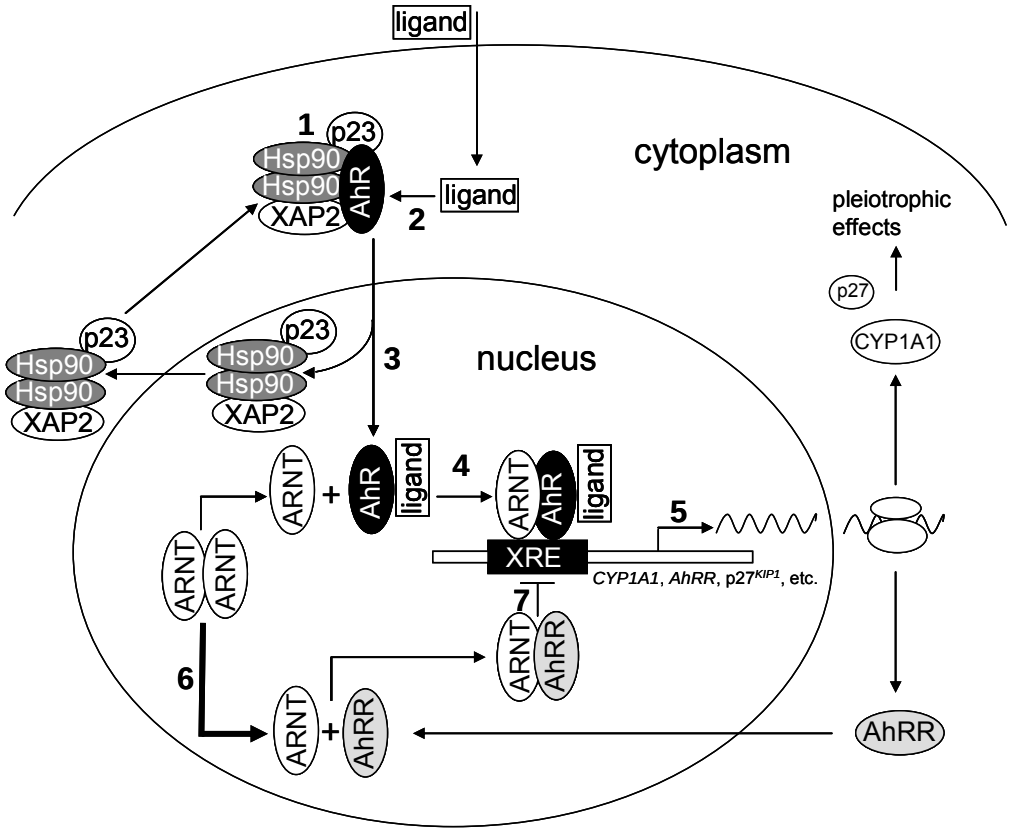


Figure 3: Model for the aryl hydrocarbon receptor signalling pathway in the presence of exogenous ligands

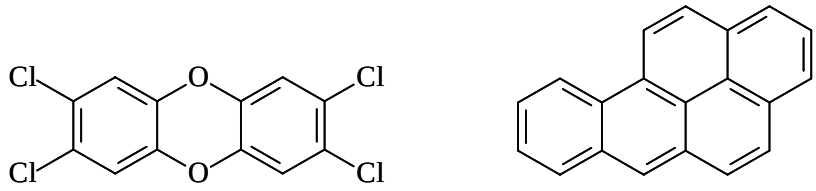
The aryl hydrocarbon receptor (AhR) translocates into the nucleus and transactivates target genes after ligand binding. HSP90: 90 kDa heat shock protein, XAP2: immunophilin homolog, p23: HSP90 associated co-chaperone, ARNT: AhR nuclear translocator, AhRR: AhR repressor, XRE: xenobiotic responsive element, CYP1A1: Cytochrome P450 1A1. Numbers refer to explanations in the text above. Adapted from Mimura and Fujii-Kuriyama (2003) (Mimura and Fujii-Kuriyama, 2003).

1.2.3 Ligands of the Aryl Hydrocarbon Receptor

The AhR can be activated by numerous structurally diverse ligands. The best characterized are the so-called classical ligands. They comprise a wide variety of ubiquitous planar hydrophobic environmental substances such as polycyclic and halogenated polycyclic aromatic hydrocarbons (PAHs, HAHs), including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Denison and Nagy, 2003) (figure 4). The metabolically more stable HAHs exhibit a very high affinity for the AhR which is in the picomolar to nanomolar range, whereas the PAHs have a significantly lower affinity in the high nanomolar to micromolar range. However, all these ligands are hydrophobic, planar, and of nearly the same size. These properties facilitate interaction with the hydrophobic ligand-binding pocket of the AhR which preferentially accepts planar ligands with maximal dimensions of 14 Å x 12 Å x 5 Å (Denison et al., 2002).

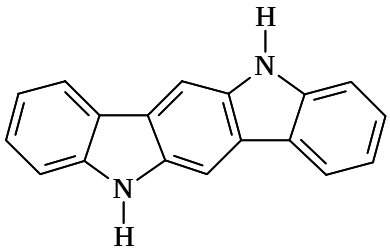
Besides these classical AhR ligands, there are numerous molecules (so-called non-classical ligands) (figure 4) including flavonoids, carotinoids, and phenolics, which can activate the AhR despite of having a very low affinity in the middle to high micromolar range (Nguyen and Bradfield, 2008). Moreover, they show a very high variety in their structural properties (Denison and Heath-Pagliuso, 1998). Dietary sources contain the major class of these AhR ligands to which humans are exposed. For instance extracts of a large variety of different vegetables, fruits, natural herbal products, and teas commonly contain AhR agonists or antagonists (Ciolino et al., 1999; Amakura et al., 2003; Zhang et al., 2003), but also molecules that are readily converted into AhR ligands (Denison and Nagy, 2003). Conversion of dietary indoles and tryptophan in the mammalian digestive tract can lead to the formation of potent AhR ligands. Indole-3-carbinole (IC3) derivatives for example induce classical AhR-mediated monooxygenases (Loub et al., 1975) and IC3 is converted to higher order AhR agonists in the acidic environment of the stomach (Bradfield and Bjeldanes, 1987; Bjeldanes et al., 1991).

Apart from its role in the induction of xenobiotic metabolism, it has long been suggested that in the absence of exogenous ligands, the AhR is also required for endogenous functions critical to life processes (Nebert et al., 1984). The existence of endogenous AhR ligands has been suggested by numerous studies in which the AhR signalling pathway is active in the absence of exogenous ligands (Hankinson et al., 1985; Ma and Whitlock, 1996; Singh et al., 1996; Weiss et al., 1996; Chang and Puga, 1998; Ito et al., 2004; Roblin et al., 2004; Shiizaki et al., 2005). The promiscuous ligand affinity of the AhR is suggestive of multiple and probably tissue-specific, endogenous ligands and in fact, several endogenous biomolecules have been identified as either binding to or activating the AhR (Denison et al., 2002). Among these ligands are for example tryptophan metabolites (Miller, 1997; Heath-Pagliuso et al., 1998) and UV-induced photoproducts of tryptophan (Rannug and Fritsche, 2006; Mukai and Tischkau, 2007). Histidine (Paine and Francis, 1980) and indigoids like indirubin and indigo (Adachi et al., 2001; Denison et al., 2006), 2-(1´H-indole-3´-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) (Song et al., 2002; Henry et al., 2006), equilenin (Ema et al., 1994; Jinno et al., 2006), arachidonic acid metabolites (Mufti and Shuler, 1996; Puga et al., 1997; Schaldach et al., 1999; Seidel et al., 2001; Kroetz and Zeldin, 2002), and the tetrapyrole heme degradation products bilirubin and biliverdin (Phelan et al., 1998) also belong to that group of ligands.

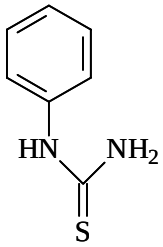


2,3,7,8-tetrachlorodibenzo-*p*-dioxin

benzo[a]pyrene



indolo-[3,2]-carbazole



phenylthiourea

Figure 4: Different AhR ligands

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and benzo[a]pyrene belong to the group of classical AhR ligands, whereas indolo-[3,2]-carbazole and phenylthiorea are non-classical AhR ligands.

1.2.4 Expression and function of the Aryl Hydrocarbon Receptor in skin and keratinocytes

Although originally characterized in liver as the major site of metabolism, the AhR-dependent signalling pathway also plays an important role in extra hepatic tissues like skin (Reiners et al., 1998), breast (Safe and Wormke, 2003), and kidney (Falahatpisheh and Ramos, 2003). AhR knockout mice exhibit numerous abnormal phenotypes. Among others, severe localized epidermal hyperplasia with hyperkeratosis and an altered expression of cytokines, keratins, and integrins was observed in the skin. Furthermore, marked dermal fibrosis and hyperproliferation of hair follicles that exhibit an abnormal orientation pattern were also evident (Fernandez-Salguero et al., 1997). These phenotypes support the involvement of the AhR in the cell homeostasis of the skin.

The mammalian skin is composed of two primary layers, the epidermis and the dermis. The epidermis is the outermost layer of the skin, consisting of predominantly keratinocytes. Hence, the epidermis is the major site of direct exposure to exogenous substances and as a model for skin, keratinocytes are the most appropriate. CYPs regulated by the AhR in human keratinocytes include CYP1A1, CYP1A2, and CYP1B1 (Reiners et al., 1998). The expression level of the AhR in proliferating keratinocytes is significantly lower than the level observed in cells derived from liver or breast tissues (Swanson, 2004). The

ability of TCDD to induce the expression level of AhR target genes correlates with the differentiation status of these cells (Jones and Reiners, 1997; Ray and Swanson, 2003). Furthermore, loss of adhesion during differentiation increased the *CYP1A1* expression level in an AhR-dependent manner (Sadek and Allen-Hoffmann, 1994; Monk et al., 2001). It is not clear whether this activation of the AhR signalling pathway is a consequence of the presence of an endogenous AhR ligand or of a mechanism that involves ligand-independent activation of the AhR. An additional phenomenon influencing AhR signalling in keratinocytes is the exposure to ultraviolet (UV) light. Exposure to UV B results in an AhR-dependent induction of the mRNA levels of *CYP1A1* and *CYP1B1* in skin (Katiyar et al., 2000; Villard et al., 2002; Fritsche et al., 2007). The high *CYP1A1* inducibility in skin and a possible impact of the AhR in epidermal cell proliferation further supports the theory that the AhR impacts on the physiological homeostasis of the skin. Expression of a constitutive active form of the AhR in keratinocytes causes inflammatory skin lesions (Tauchi et al., 2005). Moreover, there is additional evidence that the AhR plays a role in several skin conditions comprising atopic dermatitis (Tauchi et al., 2005), psoriasis (Chapman et al., 1980; Shuster et al., 1980), pigmentation disorders, skin tumour formation (Shimizu et al., 2000), and skin aging (Grady and Ernster, 1992).

1.2.5 Role of the Aryl Hydrocarbon Receptor in cell cycle progression

The AhR was originally named and identified for its function in metabolism of xenobiotic, aromatic hydrocarbons. Besides this well characterized role, the AhR is involved in the regulation of cell cycle, proliferation, apoptosis, and differentiation (Puga et al., 2002). Especially its role in cell cycle control, both via ligand-dependent and ligand-independent mechanisms remains unclear. Cell cycle progression, through the controlled process of DNA replication and cell division, is initiated in quiescent cells by mitogen stimulation. Naturally, progression of eukaryotic cells through cell cycle stages is regulated by the activities of cyclins, cyclin dependent kinases (CDKs), and cyclin dependent kinase inhibitors (CKIs), which are responsible for the ordered transition from one phase of the cycle to the next (Cheng et al., 1999; Sherr and Roberts, 1999; Smits and Medema, 2001). Their expression and activities are in turn controlled and modulated by members of the retinoblastoma protein (pRB) and E2F protein families (Coqueret, 2002; Trimarchi and Lees, 2002; Murray, 2004).

It is known that the activation of the AhR by exogenous ligands may result in an arrest of the cell cycle, including G_0/G_1 and G_2/M arrests, diminished capacity for DNA replication, inhibition of cell proliferation, apoptosis, or differentiation, depending on the cell type examined. Mechanisms of these ligands affecting a particular pathway has not been characterized. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) for instance, a prototypical AhR ligand, is able to inhibit cell proliferation by inducing a G_1 arrest in some cell lines

(Puga et al., 2002). Similar effects on the cell cycle and proliferation have been observed with other AhR ligands, e.g. benzo[a]pyrene (B[a]P) (figure 4) (Vaziri and Faller, 1997), some flavonoids (Reiners et al., 1999), and 2-(4-amino-3-methyl-phenyl)-5-fluorobezothiazole (Trapani et al., 2003). The AhR mediated G_0/G_1 arrest induced by exogenous ligands is most likely mediated by the inhibition of gene expression encoding the essential proteins for the S phase. Nevertheless, the exact molecular mechanisms still remain unclear so far.

The exact mechanism of its role in cell cycle regulation in the absence of exogenous ligands remains unclear, as well. Evidence has grown in recent years that the absence of the AhR results in prolongation of the cell cycle. For instance, Ma and co-workers found that AhR-defective Hepa-1 cells exhibit slower growth and pointed to a potential link between AhR and the G_1 phase of the cell cycle (Ma and Whitlock, 1996). Cell-type dependencies were found by studying mouse embryonic fibroblasts (MEFs) from AhR-null mice. Their reduced growth was associated with an accumulation of cells in the G_2/M phase. This arrest was mediated by an altered expression of the G_2/M kinases Cdc2 and Plk (Elizondo et al., 2000). These reports suggest that the AhR plays an endogenous role in the promotion of cell cycle progression and that this role is independent of activation by exogenous ligands. In skin and keratinocytes, almost nothing is known about this role of the AhR.

1.3 Aim of the study

Every day, our skin is exposed to numerous of xenobiotic or naturally occurring substances including AhR ligands. The best characterized function of the AhR is to regulate metabolism of xenobiotica through the induction of several phase I and phase II metabolizing enzymes, including CYP1A1. Moreover, the known physiological function of the AhR is its involvement in the regulation of cell cycle, proliferation, apoptosis, and differentiation in many tissues (Puga et al., 2002). Although contact with naturally occurring substances like eugenol and isoeugenol is almost unavoidable in our daily lives, their molecular mechanisms of action in skin is poorly understood. There are evidences supporting the hypothesis that these naturally occurring substances may impact on the AhR. Eugenol is shown to induce *CYP1A1* (Al Masaoudi et al., 2001), a well-known target gene of the AhR and furthermore, their anti-proliferative properties might also be mediated by the AhR, based on its physiological function.

Having this in mind, the aims of this thesis are twofold: on the one hand, the interrelationship between eugenol and isoeugenol, their antiproliferative properties, and the AhR signalling pathway is proved. Among others, this should provide new mechanistic insights into the molecular mechanisms of action of these two substances in skin cells. On the other hand, a comparison of cell cycle progression between wildtype and AhR knockdown keratinocytes was performed. This comparison should provide new knowledge on the physiological role of the AhR in the promotion of cell cycle progression independent of exogenous ligands.

2. Material

2.1 Chemicals, enzymes, and reagents

Benzo[a]pyrene (purity ≥ 97%), eugenol (purity ≥ 99%), and isoeugenol (purity ≥ 98%), dinitrochlorobenzene, Hoechst 33342, Ponceau-S, and the Bradford Reagent were purchased from Sigma-Aldrich (Taufkirchen, Germany) and dimethylsulfoxide (DMSO) from Carl Roth (Karlsruhe, Germany). 3'-Methoxy-4'-nitroflavone (MNF) was kindly provided by Dr. G. Vielhaber (Symrise, Holzminden, Germany). TRIZol® reagent, pre-cast SDS-PAGE gels (NuPAGE® 4-12% Bis-Tris Gels) and the protein standard MagicMark™ XP Western Standard were purchased from Invitrogen (Karlsruhe, Germany). The lysis buffer complete lysis-M EDTA free and the protein inhibitors complete Mini were from Roche Diagnostics (Mannheim, Germany). SuperSignal® West Pico was obtained from Perbio Science Deutschland GmbH (Bonn, Germany). The prestained protein standard kaleidoscope was purchased from Bio-Rad Laboratories GmbH (München, Germany). Vectashield mounting medium for fluorescence with 4´-6-Diamidino-2-phenylindole (DAPI) was obtained from Vector (Linaris Biologische Produkte GmbH, Wertheim, Germany) and reagents for flow cytometry were from BD Biosciences (Heidelberg, Germany). If not otherwise stated, all other chemicals, enzymes, and reagents were obtained from Sigma-Aldrich (Taufkirchen, Germany), Carl Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany), Applied Biosystems (Weiterstadt, Germany), Fermentas (St. Leon-Rot, Germany), Roche Diagnostics (Mannheim, Germany), Invitrogen (Karlsruhe, Germany), or Bio-Rad Laboratories GmbH (München, Germany).

2.2 Consumable materials

Consumable materials were purchased from Sarstedt (Nümbrecht, Germany), Applied Biosystems (Foster City, USA), BD Biosciences (Heidelberg, Germany), Greiner bio-one (Essen Germany), Nunc GmbH and Co. KG Thermo Fisher Scientific (Wiesbaden, Germany), and Roche Diagnostics (Mannheim, Germany) if not otherwise stated.

2.3 Cell culture

Dulbecco´s modified eagle medium (DMEM) high glucose (4.5 g/l) without L-glutamine, L-glutamine, fetal bovine serum (FBS), antibiotica/antimycotic solution, and trypsin-EDTA (0.05% / 0.02% in D-PBS) were obtained from PAA (Cölbe, Germany), geneticin from Invitrogen (Karlsruhe, Germany). The immortalized human keratinocyte cell line HaCaT was kindly provided by Prof. Dr. N. E. Fusenig (DKFZ, Heidelberg, Germany) and the

AhR knockdown HaCaT cell variant (siAhR HaCaT) by Dr. E. Fritsche (IUF, Düsseldorf, Germany).

2.4 Buffers and Solutions

If not otherwise stated, all buffers and solutions were prepared with A. deion., autoclaved after adjusting the pH-value with either NaOH or HCl and stored at ambient temperature. All buffers and solutions were prepared according to standard protocols (Russell, 2001).

2.4.1 General buffers and solutions

1 x PBS	2 mM KH_2PO_4 10 mM Na_2HPO_4/ pH 7.4 137 mM NaCl 2.7 mM KCl
50 x TAE	2 mM Tris-base 100 mM EDTA pH 8.0 (adjusted with glacial acetic acid)
1 M Tris-HCl	121.1 g/l Tris-base

2.4.2 Buffers and solutions for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Acrylamide	40% (w/v) Acrylamide/Bisacrylamide (29:1)
APS	10% (w/v) Ammonium peroxy disulfate
10 x electrophoresis buffer	250 mM Tris-base 1.9 M Glycine adjusted to pH 8.6 1% (w/v) SDS
Lower Tris	500 mM Tris-HCl, pH 8.8 0.4% (w/v) SDS

Upper Tris	1.5 M Tris-HCl, pH 6.8 0.4% (w/v) SDS

2.4.3 Buffers and solutions for western blot analysis

10x TBS	100 mM Tris-HCl, pH 8 1500 mM NaCl
TBST	1x TBS 1% (v/v) Tween-20
TBSTT	1x TBSTT 1% (v/v) Triton-X-100
20x Transfer buffer	24.22 g Tris-base 150.14 g Glycine, ad 1l with A. deion. (storage: 4°C)
1x Transfer buffer (ready to use)	50 ml 20x Transfer buffer 100 ml Methanol ad 1l with A. deion.
Stripping buffer	15 g Glycine 1 g SDS 10 ml Tween-20 pH 2.2, ad 1l with A. deion.

2.5 Oligonucleotides

Oligonucleotides were synthesized by TIB MOLBIOL (Berlin, Germany) and were supplied lyophilized. The lyophilized oligonucleotides were dissolved in sterile water (PCR grade) to an end concentration of 100 pmol/µl.

CYP1A1: 296 bp, (Mace et al., 1998)

forward: 5'-GGAGGCCTTCATCCTGGAGA-3'

reverse: 5'-CCTCCCAGCGGGCAACGGTC-3'

ARNT: 226 bp, (Vanden Heuvel et al., 1994)

forward: 5'-CGGAACAAGATGACAGCCTAC-3'

reverse: 5'ACAGAAAGCCATCTGCTGCC-3'

AhRR: 176 bp, (Yamamoto et al., 2004)

forward: 5'-ACCGCGGATGCAAAAGTAAAAG-3'

reverse: 5'-GCTCCTTCCTGCTGAGTAATTGG-3'

2.6 Antibodies

2.6.1 Primary Antibodies

Table 1: Primary antibodies used

Antigen	Species/Subclass	Dilution IF	Dilution WB	Source of supply/ Reference
$p27^{KIP1}$	goat polyclonal		1:200	Santa Cruz Biotechnology Inc. (Heidelberg, Germany)
pRB	goat polyclonal		1:200	Santa Cruz Biotechnology Inc. (Heidelberg, Germany)
CDK2	goat polyclonal		1:200	Santa Cruz Biotechnology Inc. (Heidelberg, Germany)
CDK4	goat polyclonal		1:200	Santa Cruz Biotechnology Inc. (Heidelberg, Germany)
CDK6	goat polyclonal		1:200	Santa Cruz Biotechnology Inc. (Heidelberg, Germany)
AhR	goat polyclonal	1:50		Santa Cruz Biotechnology Inc. (Heidelberg, Germany)
ARNT	rabbit polyclonal		1:200	Santa Cruz Biotechnology Inc. (Heidelberg, Germany)

2.6.2 Secondary Antibodies

Table 2: Secondary antibodies used

Antispecies	Species/Subclass	labeling	Dilution	Source of supply/ Reference
goat	bovine IgG	HRP	1:15000	Santa Cruz Biotechnology Inc. (Heidelberg, Germany)
rabbit	bovine IgG	HRP	1:15000	Santa Cruz Biotechnology Inc. (Heidelberg, Germany)
goat	rabbit IgG	Alexa Fluor 488	1:200	Molecular Probes, Invitrogen (Karlsruhe, Germany)

2.7 Reagents for reverse transcription (RT)-PCR

Reagents for reverse transcription, including RNase inhibitor (20 U/µl), MuLV reverse transcriptase (50 U/µl), $MgCl_2$ (25 mM), random hexamer primers (50 mM), 10x PCR buffer without $MgCl_2$, and dNTPs (100 mM) were obtained from Applied Biosystems (Weiterstadt, Germany).

2.8 Commercially available premixed reagents

Cell Proliferation Reagent, WST-1 (colorimetric)	Roche Diagnostics (Mannheim)
Cell Proliferation ELISA, BrDU (colorimetric)	Roche Diagnostics (Mannheim)
Light Cycler Fast Start DNA Master Kit	Roche Diagnostics (Mannheim)
SuperSignal® West Pico Chemiluminescent Substrate	Perbio Science (Bonn)
CycleTEST™ PLUS DNA Reagent Kit	BD Biosciences (Heidelberg)

2.9 Equipment

Camera	Kodak Digital Science 1D	Kodak
Centrifuges	5417 R	Eppendorf
	LC Carousel 2.0	Roche Diagnostics
	Multifuge 1 S-R	Heraeus
Clean bench	Hera Safe	Heraeus
Electrophoresis apparatus	Sub-Cell® GT	BioRad
for agarose gels	Hoefer® HE 33	Pharmacia Biotech.
Electrophoresis apparatus	Mini-PROTEAN® 3 Cell	BioRad
for PAGE	XCell Surelock™	Invitrogen

Flow cytometer	FACSCalibur	BD Biosciences
Fluorescence microscope	Orthoplan SM-Lux SN	Leica Microsystems
Haemocytometer	Blaubrand®	Brand
Homogenizer	UP50H	Dr. Hielscher GmbH
Ice-machine	AF 80	Scotsman
Incubator	Hera Cell 240	Heraeus
Light cycler	LightCycler® 2.0	Roche Diagnostics
Lumi-Imager	F1™	Roche Diagnostics
Magnetic stirrer	IKAMAG® RH	IKA Labortechnik
Microscope	Axiovert 40 CFL	Carl Zeiss AG
Microscope camera	MS 140	Intas
pH-meter	Difital pH-meter780	Metrohm
Power Supply	Power Pack P25	Biometra
Scale	BP 310S	Sartorius
Shaker	SM 25	Bühler Laborgeräte
Spectrophotometer	Synergy™ HT	BioTek
Thermal cycler	Gene Amp® 9700	Applied Biosystems
	Palm Cycler™	Corbett Research
Thermomixer	Thermomixer Compact	Eppendorf
UV-Illuminator	TFX-35 M	Life Technologies
Vortexer	VX 100	Labnet

3. Methods

All methods which are not mentioned or described were performed according to standard protocols (Russell, 2001) or as described by the manufacturer.

3.1 Cell Culture

3.1.1 Cultivation of HaCaT cells

HaCaT cells were cultured in Dulbecco´s modified eagle medium (DMEM) high glucose (4.5 g/l), supplemented with 2 mM L-glutamine, 10% heat inactivated FBS, 1% antibiotica/antimycotic solution (Boukamp et al., 1988) and for selection of siAhR HaCaT cells, 1.2% geneticin was added to the culture medium (Kalmes et al., 2006). Cells were maintained under standard culture conditions at a temperature of 37°C and an atmosphere of 5% CO_2. Subculture was performed routinely twice a week and cells were grown up to a maximum of 80% confluency. To determine the cell numbers, cells were counted under a microscope (Axiovert 40 CFL, Carl Zeiss AG, Göttingen, Germany) using a hemocytometer.

3.1.2 Cryoconservation of HaCaT cells

Cell culture medium was renewed one day before freezing and cell aliquots ($1x10^6$ cells/ml) were frozen in cryo-vials in FBS containing 10% DMSO, for 2 h at -20°C, overnight at -80°C and for longtime storage transferred to liquid nitrogen.

3.1.3 Incubation of HaCaT cells with eugenol and isoeugenol

If not otherwise stated, HaCaT cells were seeded in a density of $0.2x10^5$ cells/cm^2 and after 4 h, eugenol and isoeugenol were added to the culture medium. Cells were grown in the presence of both substances for up to 48 h. For synchronization of HaCaT cells in the G_0/G_1 phase of the cell cycle, cells were seeded, cultured for 24 h, and then synchronized by serum starvation (0.2% FBS). Serum was readded after 24 h and cells were incubated in the presence of either eugenol or isoeugenol for 24 h. When performing an additional incubation with the AhR antagonist 3´-methoxy-4´-nitroflavone (MNF) cells were preincubated with either 10 µM MNF (HaCaT) or 1 µM MNF (siAhR HaCaT) for 1 h before adding the compounds. Both eugenol and isoeugenol and in addition all positive controls used within this thesis were diluted in DMSO. Therefore, DMSO was used as a negative control in every experiment.

3.2 Cell viability

Propidium iodide (PI) staining was used to determine membrane integrity as a parameter for necrotic cell death. PI is excluded from cells with intact cell membranes and intercalates into the DNA of cells with membrane damages. HaCaT cells ($5x10^5$) were plated in 25 cm^2 culture flasks and incubated with medium alone, the solvent DMSO alone (1%, negative control), DNCB (10 µM, positive control), or different concentrations of either eugenol or isoeugenol (60 µM, 150 µM, 300 µM, and 600 µM) for 48 h. After stimulation, 10 µl (1 mg/ml in H_2O) PI were added directly to the culture medium for 5 min. Non-adherent cells were collected from cell culture supernatants by centrifugation (5 min, 300 x g) and adherent cells were detached with trypsin-EDTA. Both cell fractions were pooled, washed with PBS, and resuspended in PBS for analysis of $1x10^4$ cells via the FACSCalibur flow cytometer. Data analysis was performed with the software CellQuest™ Pro (Becton Dickinson, Heidelberg, Germany).

3.3 Immunocytochemistry

In order to determine the intracellular localization of the aryl hydrocarbon receptor after incubation with eugenol and isoeugenol, immunocytochemistry was performed. Therefore, HaCaT cells ($34x10^3$ cells per chamber) were seeded in chamber slides (Nunc GmbH and Co. KG Thermo Fisher Scientific, Wiesbaden, Germany) and cultivated for 48 h. Subsequently, cells were treated for 90 min with eugenol (600 µM), isoeugenol (300 µM), benzo[a]pyrene (10 µM, positive control), and the solvent DMSO alone (1%). After washing the slides three times with PBS, cells were fixed with ice cold methanol:aceton (7:3) for 5 min at -20°C (Kruse et al., 2006) and rinsed three times with PBS. Blocking solution (PBS, 1% BSA) was added and samples were incubated for 30 min at ambient temperature. Cells were then incubated with the primary antibody (goat polyclonal anti-AhR, 1:50, Santa Cruz Biotechnology Inc, Heidelberg, Germany) over night at 4°C. After washing the cells three times with PBS, the secondary antibody (rabbit anti goat IgG alexa fluor 488, 1:200, Molecular Probes; Invitrogen, Karlsruhe, Germany) was applied and incubated for 1 h at room temperature. Antibody dilutions were made in blocking solution. Subsequently, the cells were rinsed three times with PBS, once with A. deion., mounted using mounting medium vectashield for fluorescence with DAPI (Vector; Linaris Biologische Produkte GmbH, Wertheim, Germany), and analyzed with a fluorescence microscope (Orthoplan SM-Lux SN, Leica Microsystems, Wetzlar, Germany, 400x augmentation) equipped with a microscope camera (Intas, Göttingen, Germany). Images were collected by using the software Image-Pro Plus 5.0 (MediaCybernetics, Germany).

3.4 Gene expression analysis

3.4.1 RNA isolation

Total RNA of HaCaT cells was isolated with the TRIZol® reagent according to the manufacturer's instructions based on the method of (Chomczynski and Sacchi, 1987). RNA concentration was determined by measuring the absorbance at 260 nm in a spectrophotometer. 1 µg RNA was immediately used for reverse transcription and the remaining part was quick-frozen in liquid nitrogen and stored at -80°C.

3.4.2 Reverse transcription (RT) PCR

cDNA was synthesized using 1 µg of total RNA in a total volume of 10 µl containing 25 µM dNTPs, 5 mM $MgCl_2$, 2.5 µM random hexamer primers, 1 U/µl RNase inhibitor, and 25 U/µl MuLV reverse transcriptase. RT-PCR approaches were incubated at the following temperatures using a thermal cycler (Palm Cycler ™, Corbett Life Science, Wasserburg Bodensee, Germany): 22°C for 10 min, 42°C for 15 min, 99°C for 5 min, 5°C for 5 min. In order to exclude DNA contaminations, respective reactions without MuLV reverse transcriptase, were set up.

3.4.3 Quantitative real-time PCR

Quantitative real-time PCR for *CYP1A1*, *ARNT*, and *AhRR* mRNA was performed in glass capillaries with the LightCycler™ instrument (Roche Diagnostics; Mannheim, Germany). The PCR reactions were set up using the LightCycler-FastStart DNA Master SYBR Green® I kit according to the manufacturer's instructions. Briefly, 2 µl cDNA solution, $MgCl_2$ (*CYP1A1*: 4 mM; *ARNT*: 2 mM, *AhRR*: 2 mM), specific primers, and PCR grade water were added to 1 µl 10x SYBR Green® FastStart Master Mix up to a final volume of 10 µl. The temperature profiles included an initial denaturation step at 95°C for 10 min followed by 50 amplification cycles (*CYP1A1*: denaturation: 95°C for 1 s, annealing: 62°C for 5 s, elongation: 72°C for 13 s; *ARNT*: denaturation: 95°C for 1 s, annealing: 64°C for 10 s, elongation: 72°C for 20 s, *AhRR:* denaturation: 94°C for 5 s, annealing: 60°C for 5 s, elongation: 72°C for 6 s). Ramp rates were set to 20°C per s. SYBR Green® I fluorescence was measured at the end of each elongation cycle. The specificity of the PCR amplification was confirmed by melting curve analysis (*CYP1A1*: T_m = 86°C; *ARNT*: T_m = 84°C, *AhRR*: T_m = 84°C) and initially aliquots of the PCR products were separated on 2% agarose gels. Quantification of the unknown amounts of the respective gene products was carried out by using external standards. *ARNT* was used as housekeeping gene. Data was analyzed with the LightCyclerSoftware 4.05 from

Roche Diagnostics (Mannheim, Germany) and calculated as fold induction (stimulus/vehicle control of cDNA/ µg total RNA [fg/µg]).

CYP1A1, *ARNT,* and *AhRR* standards were generated by PCR amplification using a thermal cycler (Palm Cycler ™, Corbett Life Science, Wasserburg Bodensee, Germany) from cDNA templates with the above mentioned primers as follows: 3 µl of cDNA template was amplified in a total volume of 100 µl reaction consisting of 100 pmol of each primer, 2 mM dNTPs, 10 µl 10x PCR buffer containing 15 mM $MgCl_2$, and 2.5 U Ampli Taq Gold®. The experimental protocol included an initial denaturation step at 95°C for 10 min followed by 40 amplification cycles (*CYP1A1*: denaturation: 95°C for 30 s, annealing: 62°C for 30 s, elongation: 72°C for 1 min; *ARNT*: denaturation: 95°C for 30 s, annealing: 64°C for 30 s, elongation: 72°C for 1 min, *AhRR*: denaturation: 95°C for 30 s, annealing: 60°C for 30 s, elongation: 72°C for 1 min). After amplification, the resulting DNA fragments were purified with MicroSpin™ S-300 HR columns (Amersham Pharmacia Biotech Europe; Freiburg, Germany). The concentration was determined by measuring the absorbance at 260 nm. The purified cDNA fragment of *ARNT* was cloned into the vector pCR®II-TOPO® (Invitrogen, Karlsruhe, Germany).

3.5 Cell Cycle studies

To analyze the cell cycle state distribution of HaCaT cells, cells ($5x10^5$) were plated in 25 cm^2 culture flasks. Cells were cultivated with medium alone or incubated with eugenol (60 µM, 300 µM, and 600 µM) and isoeugenol (60 µM, 150 µM, and 300 µM), respectively, or solvent DMSO alone (1%, negative control) for 48 h. When performing a co-treatment with MNF, cells were preincubated as described in section 3.1.2. After detachment with trypsin-EDTA the different phases of the cell cycle were evaluated after staining the DNA with propidium iodide (PI), provided with the CycleTEST™ PLUS DNA Reagent Kit, according to the manufacturer's instructions. Analysis was performed via FACSCalibur flow cytometer using the software CellQuest™Pro (BD Biosciences, Heidelberg, Germany). For cell cycle analysis with synchronized HaCaT cells, cells ($5x10^5$) were plated in 25 cm^2 tissue flasks, cultured for 24 h, and then synchronized in G_0/G_1 by serum starvation (0.2% FBS) for 24 h. Subsequently, 10% FBS was readded simultaneously with the application of either eugenol (600 µM) or isoeugenol (300 µM), and cells were incubated for 24 h. The percentages of cells in each stage of the cell cycle were analyzed as described above.

3.6 Cell Proliferation Studies

3.6.1 BrdU incorporation assay

In order to determine the rate of DNA synthesis of HaCaT cells, a 5‘-bromo-2‘-deoxyuridine (BrdU) incorporation assay was used (Gratzner, 1982; Iwakiri et al., 2004). BrdU is an analogue of thymidine and incorporates into the DNA during DNA synthesis. Cells (5.4 x 10^3 cells/well) were seeded in 96 well plates, and incubated for 48 h with different concentrations of eugenol and isoeugenol (60 µM, 150 µM, 300 µM, 600 µM), respectively, or the solvent DMSO alone (1%, negative control). BrdU (final concentration 100 µM) was added to the cells for the last 24 h of the incubation. Incorporation of BrdU was determined with a spectrophotometer using the Cell Proliferation ELISA, BrdU (colorimetric) according to the manufacturer's instructions at a wavelength of 370 nm with a reference wavelength of 492 nm.

3.6.2 Wst-1 assay

The metabolic activity of HaCaT cells was determined by using a colorimetric assay based on the reduction of a tetrazolium salt, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) to water soluble formazan (Mosmann, 1983; Huang et al., 2004). In brief, HaCaT cells (5.4 x 10^3 cells/well) were seeded in 96 well plates and incubated for 48 h with different concentrations of eugenol and isoeugenol (60 µM, 150 µM, 300 µM, 600µM), respectively, or the solvent DMSO alone (1%, negative control). Wst-1 reagent was added to the cells for the last 2 h of incubation. The formazan dye produced by metabolically active cells was measured at 450 nm by a spectrophotometer with a reference wavelength of 655 nm.

3.7 Preparation of cell lysates

HaCaT cells ($1.5x10^6$) were plated in 75 cm^2 flasks and cultured for 24 h. Subsequently, cells were synchronized and incubated with eugenol and isoeugenol as described above. After detachment with trypsin-EDTA, cells were washed twice with PBS, lysed with the lysis buffer complete lysis-M EDTA-free containing protease inhibitors (complete Mini EDTA free) (Roche Diagnostics, Mannheim, Germany), and sonicated 8 s four times on ice. Clear lysates were prepared by centrifugation at 20.000x g for 10 min at 4°C. Protein content was determined by using the Bradford Reagent according to the manufacturer's instructions.

3.8 SDS-PAGE

Separation of proteins on the basis of their molecular weights was performed with discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Within this thesis, pre-cast SDS gels 4-12% or mini-gels with 12% polyacrylamide were used. Mini-gels were prepared as follows.

Table 3: Composition of separating gel (12%) and stacking gel

Solution	Separating gel [ml]	Stacking gel [ml]
A. deion.	2.15	1.2
Acrylamide	1.5	0.25
Lower Tris	1.25	-----
Upper Tris	-----	0.5
10% SDS	0.05	0.02
10% APS	0.05	0.02
Temed	0.005	0.002

100 µg (12% gel) of dissolved protein was mixed with an equal volume of sample buffer (Roti®-Load 1), boiled for 5 min, and subjected to SDS-PAGE. An electric current of 15 mA was applied across the gel during migration of proteins through the stacking gel and after entering the resolving gel, electric current was arisen to 30 mA. When using 4-12% pre-cast gels, 25 µg of dissolved protein was mixed with sample buffer 3:1 (4x NuPAGE® LDS Sample Buffer) including reducing agent (NuPAGE® Reducing Agent (10 x)), heated at 70°C for 10 min, and loaded on the gel. Voltage was constantly at 200 V.

3.9 Western blot

Following the separation of proteins in a discontinuous SDS-PAGE, the proteins were electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane at a constant electric current of 400 mA for one and a half hour by using a wet blot system. After transfer, membranes were stained with Ponceau-S according to the manufacturer's instructions, to ensure equal protein loading. Subsequently, membranes were blocked with 5% non-fat dry milk in TBST for 30 min at ambient temperature. The membrane was then incubated with the

desired primary antibody for 1 h at room temperature. After washing the membrane three times in TBSTT and once in TBST, the blot was incubated for 30 min with the appropriate peroxidase-conjugated secondary antibody. The membrane was washed four times in TBST, once in TBS, and developed with SuperSignal® West Pico Chemiluminescent Substrate (Perbio Science Deutschland GmbH, Bonn, Germany) according to the manufacturer's instructions. Protein expression was detected by using the Lumi-Imager from Roche Diagnostics (Mannheim, Germany) and the software LumiAnalyst. In some cases, membranes were stripped and reprobed with anti-ARNT antibody as an additional loading control. Therefore, the membrane was washed with A. deion. and stored at -20°C or subsequently regenerated. For regeneration, the membrane was washed twice in stripping buffer and twice in PBS. After washing the membrane once in TBST, the membrane was reactivated in methanol and subsequently blocked with 5% non-fat dry milk in TBST for 30 min at ambient temperature. Antibody incubation and detection of protein expression was performed as described above.

3.10 Statistical analysis

Differences between controls and treatment groups were determined by using the Student's *t*-test. A *p*-value <0.05 was considered to be statistically significant.

4. Results

4.1 Effects of eugenol and isoeugenol on the viability of HaCaT cells

When studying physiological influences of a chemical substance it is important to exclude cytotoxic effects caused by these chemicals. In order to investigate the range of non-toxic concentrations of eugenol and isoeugenol, cell viability was determined by propidium iodide (PI) staining. PI is excluded from cells with intact cell membranes and intercalates into the DNA of cells with membrane damages. In brief, HaCaT cells were treated with varying concentrations of either eugenol or isoeugenol (60 µM, 150 µM, 300 µM, and 600 µM) for 48 h. Untreated cells function as negative control. For the purpose of excluding cytotoxic effects of the solvent, cells were also treated with DMSO alone (1%). Dinitrochlorobenzene (DNCB, 10 µM), known for its cytotoxicity on HaCaT cells (Van Och et al., 2005), served as positive control. As shown in figure 5, neither for eugenol (figure 5A) nor for isoeugenol (figure 5B) significant effects were detected (n=3). Only 600 µM isoeugenol reduced the cell viability to approximately 70%. Hence, in all following experiments 600 µM was used as the highest concentration for eugenol and 300 µM as the highest concentration for isoeugenol. The solvent DMSO alone did not influence cell viability.

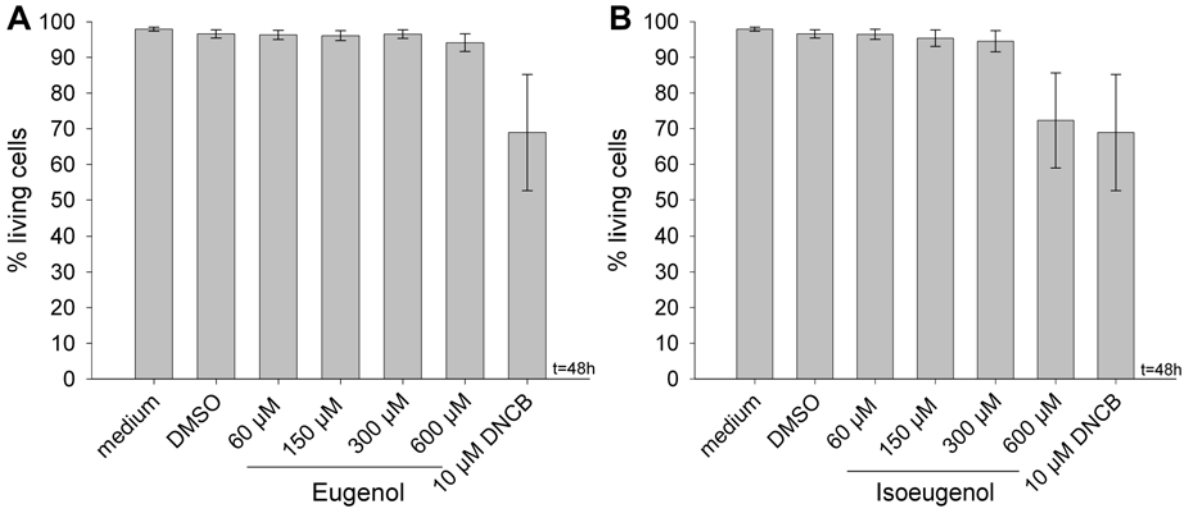


Figure 5: Effects of eugenol and isoeugenol on the viability of HaCaT cells

HaCaT cells were cultivated in medium alone, in the presence of the solvent DMSO (1%), or varying concentrations of either eugenol or isoeugenol (60 µM - 600 µM) for 48 h. Dinitrochlorobenzene (DNCB, 10 µM) served as positive control. Cells were stained with propidium iodide and analyzed via flow cytometry to determine the percentages of living cells. Bars represent the mean of three independent experiments +/- SEM. (A) Incubation with eugenol. (B) Incubation with isoeugenol.

4.2 Impact of eugenol and isoeugenol on the intracellular localization of the AhR in HaCaT cells

One focal point of this thesis is to study whether the molecular mechanisms of action of eugenol and isoeugenol are mediated through the aryl hydrocarbon receptor. Previous investigations of our working group showed that eugenol can induce *CYP1A1* mRNA in primary human keratinocytes (Al Masaoudi et al., 2001). Given that the regulation of *CYP1A1* expression is mediated through the AhR after ligand binding and translocation into the nucleus, the intracellular localization of the AhR in HaCaT cells was determined after treatment with eugenol and isoeugenol. Therefore, HaCaT cells were incubated with either eugenol (600 µM) or isoeugenol (300 µM) for 90 min. DMSO (1%) served as negative control. Benzo[a]pyrene (B[a]P, 10 µM), a well-known ligand of the AhR (Nguyen and Bradfield, 2008), served as positive control. Figure 6A demonstrates that in cells treated with DMSO the AhR is predominantly localized in the cytoplasm, whereas after eugenol and isoeugenol treatment the major part of AhR protein is translocated into the nucleus (figure 6 B and C). After incubation with the positive control B[a]P, the AhR is also predominantly localized in the nucleus (figure 6D).

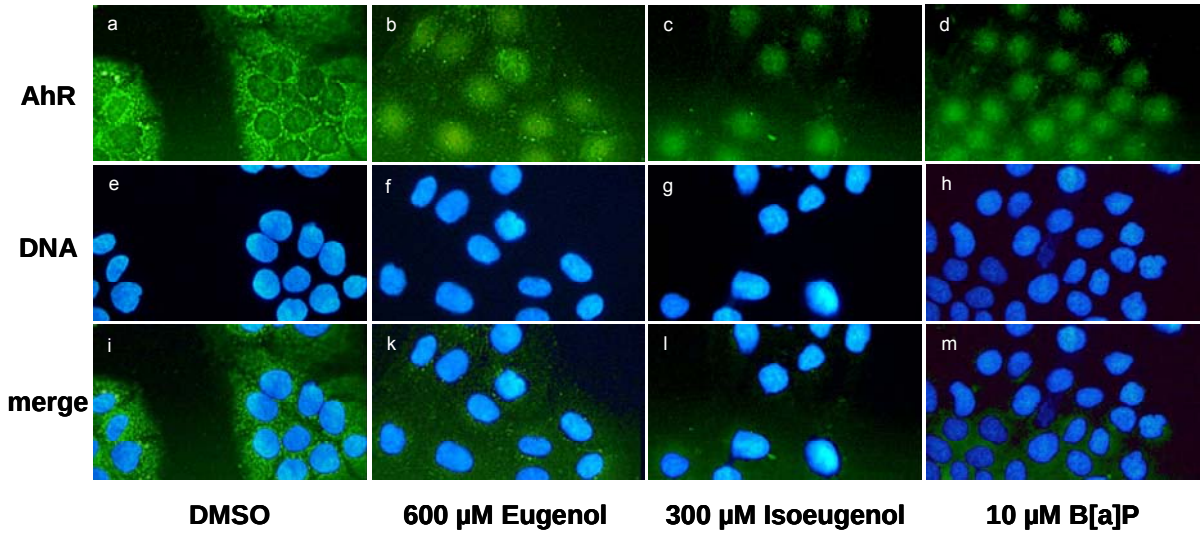


Figure 6: Impact of eugenol and isoeugenol on the localization of AhR in HaCaT cells

HaCaT cells were incubated with either the solvent DMSO alone (1%, negative control), eugenol (600 µM), isoeugenol (300 µM), or benzo[a]pyrene (B[a]P, 10 µM, positive control) for 90 min. The localization of the AhR was determined by fluorescence microscopy (400x augmentation) (a-d, i-m). Total DNA was stained with dapi (e-h, i-m).

4.3 Effects of eugenol and isoeugenol on AhR target genes in HaCaT cells

In order to support the findings concerning the translocation of the AhR into the nucleus, the impact of eugenol and isoeugenol on the expression of AhR target genes in HaCaT cells was studied. Therefore, *CYP1A1* and *AhRR* expression was determined by measuring the steady state mRNA levels with quantitative real-time PCR. *ARNT* served as housekeeping gene. Cells were treated with either eugenol (600 µM) or isoeugenol (300 µM) for 24 h. DMSO (1%) served as negative control and B[a]P (10 µM), a known inductor of the AhR target genes, served as positive control. As demonstrated in figure 7 a moderate but statistically significant *CYP1A1* (figure 7A) *and AhRR* (figure 7B) mRNA induction was found after incubation with eugenol and isoeugenol. For *CYP1A1* 600 µM eugenol caused a 7 fold increase (n=5, SEM=1.4, $p<0.001$) and 300 µM isoeugenol caused a 4 fold increase (n=4, SEM=1.4, $p<0.05$) compared to control cells. For *AhRR* 600 µM eugenol caused a 6 fold increase (n=3, SEM=0.9, $p<0.05$), whereas 300 µM isoeugenol caused a 4 fold induction (n=3, SEM=1.9), compared to control cells. *ARNT* mRNA levels (figure 7C) were not significantly changed after incubation with the mentioned substances. In the absence of the substances mRNA levels of all three genes were detectable and the presence of DMSO alone did not modify these mRNA levels. The positive control B[a]P induced *CYP1A1* mRNA by 166 fold (n=3, SEM=15.8, $p<0.001$), *AhRR* mRNA by 30 fold (n=3, SEM=7.2, $p<0.05$), and *ARNT* mRNA levels remained unchanged.

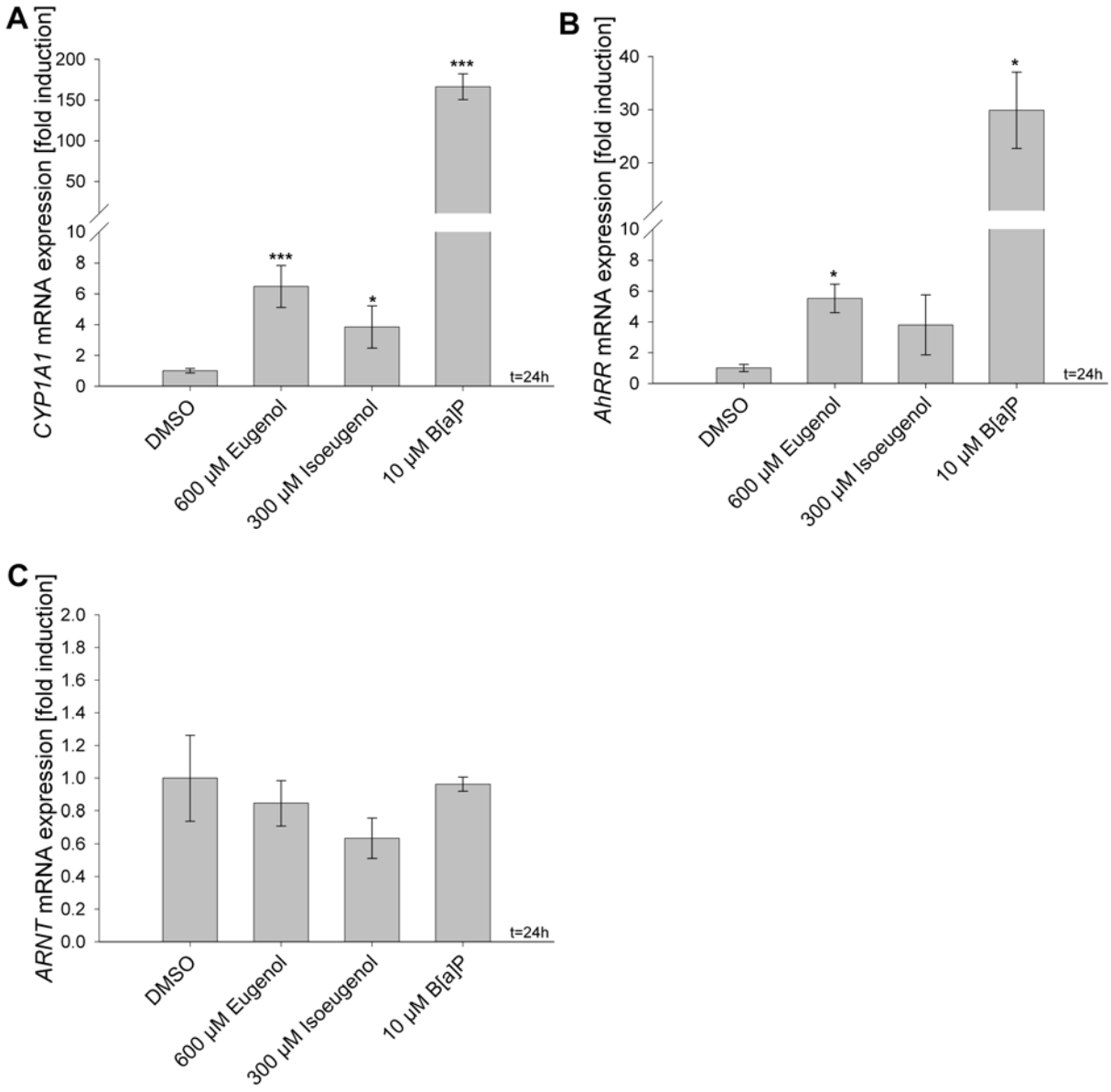


Figure 7: Effects of eugenol and isoeugenol on AhR target genes in HaCaT cells

HaCaT cells were incubated with either the solvent DMSO alone (1%, negative control), eugenol (600 µM), isoeugenol (300 µM), or benzo[a]pyrene (B[a]P, 10 µM, positive control) for 24 h. Total RNA was isolated, reverse transcribed and mRNA levels of *CYP1A1*, *AhRR*, and *ARNT* were determined by quantitative real-time PCR. Bars represent the mean of at least three independent experiments +/- SEM and asterisks indicate significant differences from DMSO treated controls (*p<0.05, **p<0.01, ***p<0.001). Data was analyzed with the LightCyclerSoftware 4.05 from Roche Diagnostics (Mannheim, Germany) and calculated as fold induction (stimulus/vehicle control of cDNA/ µg total RNA [fg/µg]).

4.4 Influence of eugenol and isoeugenol on cell cycle progression in HaCaT cells

4.4.1 Influence of eugenol and isoeugenol on cell cycle state distribution

The ligand activated AhR is known for its impact on cell cycle progression in various cell types. Moreover, eugenol and isoeugenol are known for their antiproliferative properties. In skin, little is known about the physiological function of the AhR. In order to investigate a possible role of eugenol and isoeugenol on cell cycle progression in HaCaT, cells were incubated with distinct concentrations of eugenol (60 µM, 300 µM, and 600 µM) and isoeugenol (60 µM, 150 µM, and 300 µM) for 48 h. DMSO (1%) served as negative control. Subsequently, cells were subjected to cell cycle analysis by flow cytometry. As demonstrated in figures 8A and 8B both compounds caused an accumulation of cells in the G_0/G_1 phase of the cell cycle. The highest effects for eugenol (600 µM) yielded 12% more cells in the G_0/G_1 phase (n=3, SEM=1.9, $p<0.05$) compared to solvent treated cells. Isoeugenol induced this accumulation to a stronger extent; 300 µM isoeugenol already resulted in 16% (n=3, SEM=2.7, $p<0.05$) more cells in the G_0/G_1 phase compared to control cells. These results were accompanied by corresponding lower percentages of cells in the S and G_2/M phases. In order to expand knowledge about this fragrance-induced cell cycle arrest, synchronized HaCaT cells were analyzed after incubation with eugenol and isoeugenol. This approach was performed to verify whether both substances are able to impact on already arrested cells and inhibit cell cycle progression after serum readdition. Cycling cells in culture will enter a quiescent (G_0) state upon withdrawal of serum from growth medium. Serum stimulation triggers a synchronized reentry into the G_1 phase of the cell cycle. Cell cycle synchronization was thus achieved by serum starvation for 24 h causing an accumulation of HaCaT cells in the G_0/G_1 phase of the cell cycle. After serum was readded, cells were incubated in the presence or absence of eugenol (600 µM), isoeugenol (300µM), or the solvent DMSO alone (1%) for either 24 h or 48 h. Finally, cells were subjected to flow cytometry. Figure 8C demonstrates that cells incubated with eugenol for 24 h exhibited 32% (n=3, SEM=4.2, $p<0.01$) more cells in the G_0/G_1 phase, whereas the presence of isoeugenol resulted in 34% (n=3, SEM=6.0, $p<0.05$) more cells in the G_0/G_1 phase when compared to control cells. Corresponding lower percentages of cells in the S and G_2/M phases were detected. After 48 h (figure 8D) only marginal effects on cell cycle progression were observed.

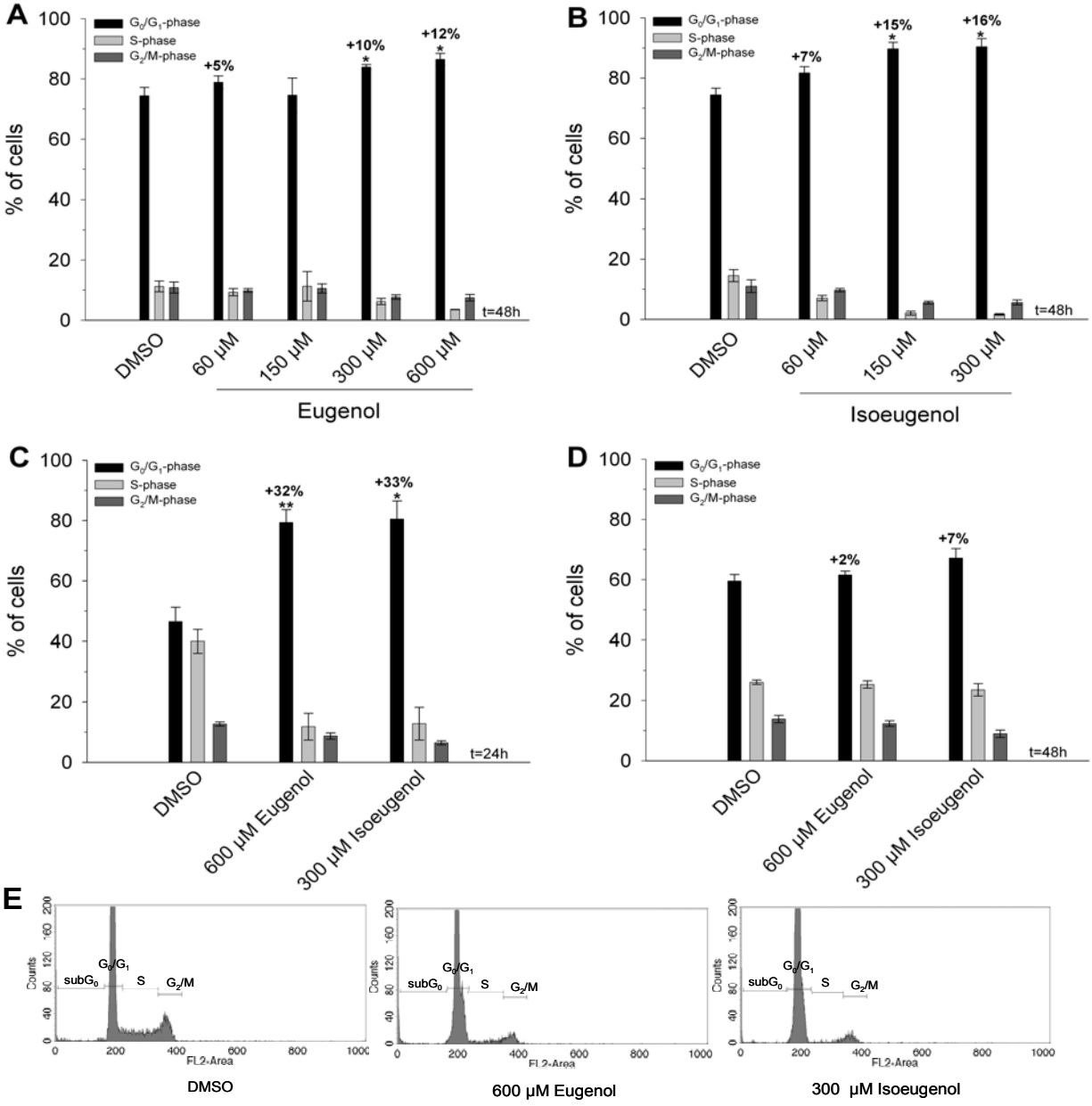


Figure 8: Effects of eugenol and isoeugenol on cell cycle state distribution in HaCaT cells

HaCaT cells were cultivated in the presence of the solvent DMSO alone (1%, negative control) and varying concentrations of eugenol (60 µM - 600 µM) or isoeugenol (60 µM - 300 µM), for either 24 h or 48 h. Cell cycle state distribution was analyzed via flow cytometry to determine the percentage of cells in the G_0/G_1, S, and G_2/M phases. Bars represent the mean of three independent experiments +/- SEM and asterisks indicate significant differences from DMSO treated controls (*$p<0.05$, **$p<0.01$). (A) Incubation of HaCaT cells with eugenol for 48 h. (B) Incubation of HaCaT cells with isoeugenol for 48 h. (C) Incubation of synchronized HaCaT cells with eugenol or isoeugenol for 24 h. (D) Incubation of synchronized HaCaT cells with eugenol or isoeugenol for 48 h. (E) Histograms of one representative experiment from (A) and (B) as analyzed with the software CellQuest^{TM} Pro: cell cycle phase distribution after 48 h.

4.4.2 Involvement of the AhR on the eugenol- and isoeugenol-induced cell cycle arrest in HaCaT cells

Knowing that eugenol and isoeugenol cause a translocation of the AhR from the cytoplasm into the nucleus and induce AhR target genes, the influence of the AhR on the eugenol/isoeugenol-induced cell cycle arrest in HaCaT cells was now investigated. Therefore, HaCaT cells were incubated with eugenol (600 µM) and isoeugenol (300 µM) in the presence of the well-known AhR antagonist 3´-methoxy-4´-nitroflavone (MNF) (Lu et al., 1995; Gasiewicz et al., 1996) for 48 h. DMSO (1%) served as negative control. In a first series of experiments 1 µM MNF was applied which is capable of suppressing AhR dependent cell cycle arrests in many circumstances (Elferink, 2003; Ray and Swanson, 2004). Here it was noted that this concentration was inefficient to suppress the eugenol/isoeugenol-induced cell cycle arrest (data not shown). In the presence of 10 µM MNF, eugenol was no longer able to arrest the cells in the G_0/G_1 phase of the cell cycle (n=3, SEM=6.2) but for isoeugenol 10 µM MNF was even not sufficient to block the G_0/G_1 arrest completely (n=3, SEM=9.2) (figure 9). Percentages of cells in the G_0/G_1 phase were only reduced from 16%, in the absence of MNF (cf. figure 8B), to 9% in the presence of MNF.

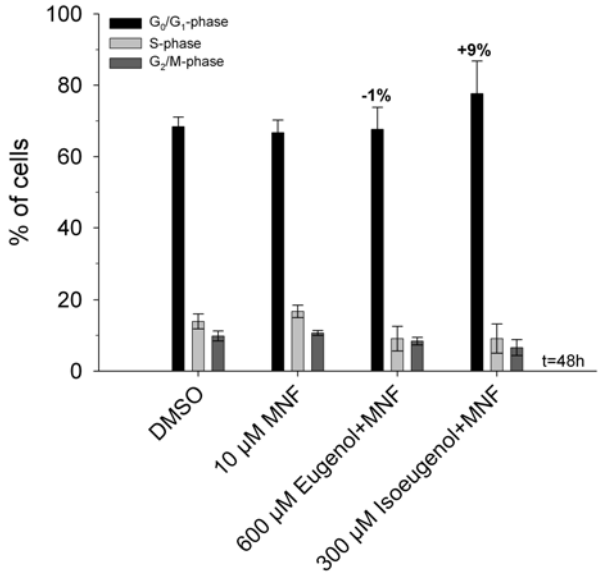


Figure 9: Influence of the AhR on the eugenol/isoeugenol-induced cell cycle arrest in HaCaT cells

HaCaT cells were incubated with the solvent DMSO alone (1%, negative control), the AhR antagonist 3´-methoxy-4´-nitroflavone (MNF, 10 µM) alone, and with eugenol (600 µM) or isoeugenol (300 µM) in the presence of MNF for 48 h. Cell cycle state distribution was analyzed via flow cytometry to determine the percentage of cells in the G_0/G_1, S, and G_2/M phases. Bars represent the mean of three independent experiments +/- SEM.

4.4.2.1 Impact of eugenol and isoeugenol on HaCaT cells expressing a siRNA targeted against the AhR

With the purpose of further confirming the impact of the AhR on the eugenol/isoeugenol-induced cell cycle arrest, the effects of these substances on cell cycle progression in a HaCaT cell line stably expressing a siRNA targeted against the AhR were studied (siAhR HaCaT). The protein level of the AhR in this cell line is reduced by 80% (Fritsche et al., 2007). In order to exclude unspecific effects of eugenol and isoeugenol on the siAhR HaCaT cells, first of all propidium iodide staining was performed with this cell line as described in section 4.1. As demonstrated in figure 10, the reduced AhR protein level in the siRNA HaCaT cells did not modify their membrane integrity in comparison to wildtype HaCaT cells (cf. figure 5). Neither for eugenol (figure 10A) nor for isoeugenol (figure 10B) statistically significant effects were detected (n=3). Untreated cells function as negative control. In order to exclude cytotoxic effects of the solvent, cells were also treated with DMSO alone (1%). DNCB served as positive control.

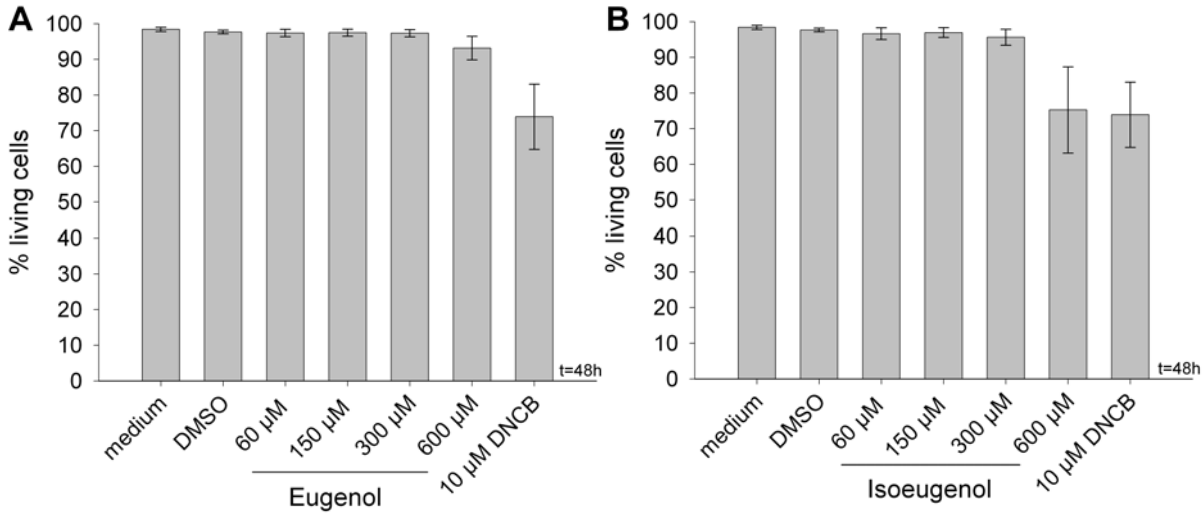


Figure 10: Effects of eugenol and isoeugenol on the viability of siAhR HaCaT cells

siAhR HaCaT cells were cultivated in medium alone, in the presence of the solvent DMSO alone (1%), or varying concentrations of either eugenol or isoeugenol (60 µM - 600 µM) for 48 h. Dinitrochlorobenzene (DNCB, 10 µM) served as positive control. Cells were stained with propidium iodide and analyzed via flow cytometry to determine the percentages of living cells. Bars represent the mean of three independent experiments +/- SEM. (A) Incubation with eugenol. (B) Incubation with isoeugenol.

In order to study cell cycle state distribution in dependence of the AhR in these variant of HaCaT cells, siAhR HaCaT cells were incubated for 48 h with different concentrations of either eugenol (60 µM, 300 µM, and 600 µM) or isoeugenol (60 µM, 150 µM, and 300 µM). Subsequently, cell cycle phase distribution was analysed by flow cytometry. As demonstrated in figures 11A and 11B, both compounds (eugenol 60 µM – 600 µM; isoeugenol 60 µM - 300 µM) caused an accumulation of cells in the G_0/G_1 phase of the cell cycle depending on their dose. The highest effects for eugenol yielded 18% more cells in the G_0/G_1 phase ($n=3$, SEM=1.2, $p<0.01$), while 300 µM isoeugenol already resulted in 23% ($n=3$, SEM=1.0, $p<0.001$) more cells in the G_0/G_1 phase when compared to solvent treated cells. These results were accompanied by corresponding lower percentages of cells in the S and G_2/M phases. As demonstrated in figure 11C, the presence of the AhR antagonist 3´-methoxy-4´-nitroflavone (MNF) reduced the eugenol/isoeugenol-induced cell cycle arrest. MNF alone did not exhibit any impact on cell cycle progression in siAhR HaCaT. Under these conditions 1 µM MNF was sufficient to reduce the eugenol/isoeugenol-induced G_0/G_1 arrest.

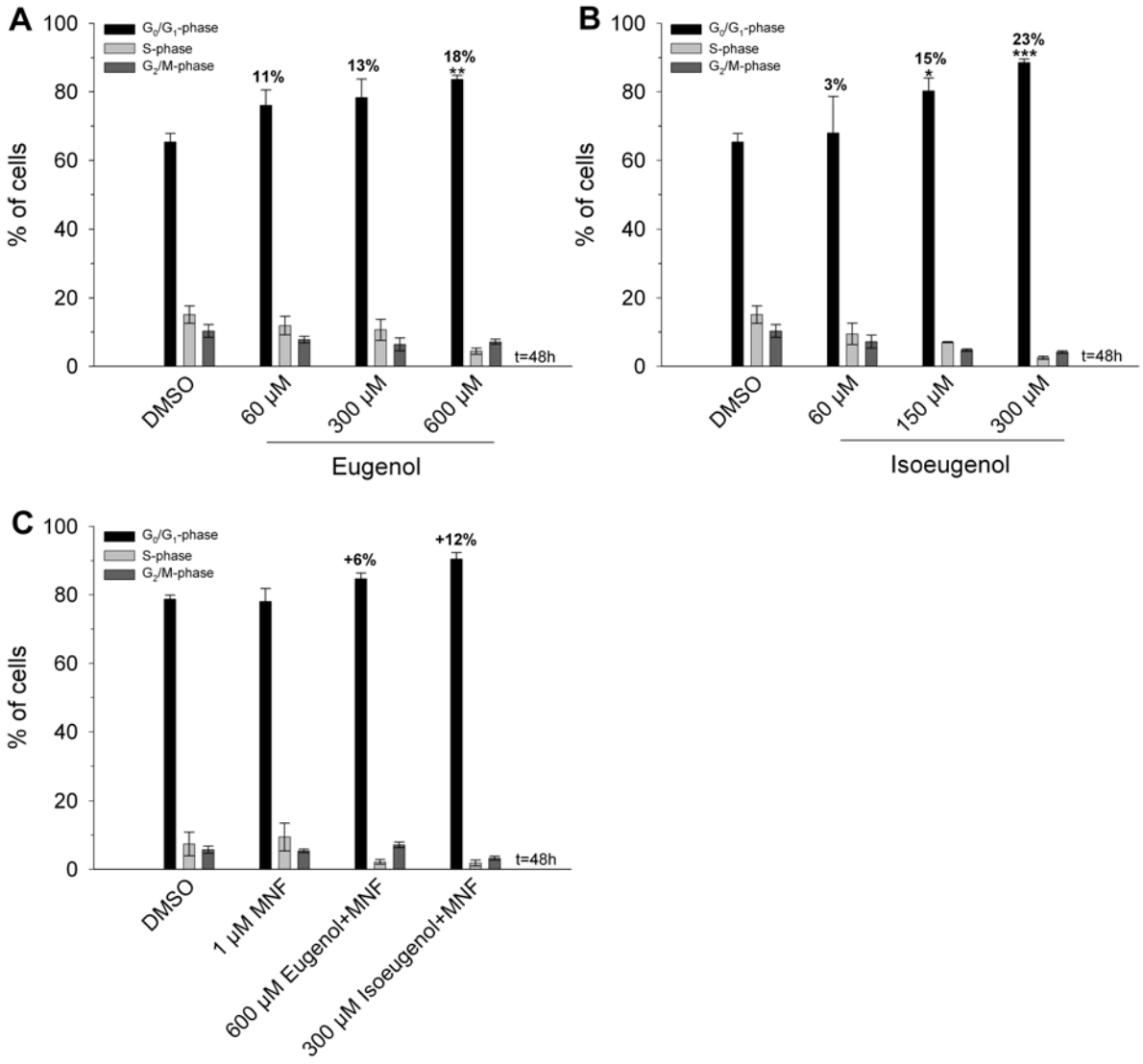


Figure 11: Influence of eugenol and isoeugenol on cell cycle state distribution in siAhR HaCaT cells

siAhR HaCaT cells were cultivated in the presence of the solvent DMSO alone (1%, negative control) and varying concentrations of eugenol (60 µM - 600 µM) or isoeugenol (60 µM - 300 µM) alone, and in the presence of the AhR antagonist 3´-methoxy-4´-nitroflavone (MNF, 1 µM) for 48 h. Cell cycle state distribution was analyzed via flow cytometry to determine the percentage of cells in the G_0/G_1, S, and G_2/M phases. Bars represent the mean of three independent experiments +/- SEM and asterisks indicate significant differences from DMSO treated controls (*$p<0.05$, **$p<0.01$, ***$p<0.001$). (A) Incubation of siAhR HaCaT cells with eugenol. (B) Incubation of siAhR HaCaT cells with isoeugenol. (C) Incubation of siAhR HaCaT cells with eugenol (600 µM) or isoeugenol (300 µM) in the presence of MNF.

4.4.3 Effects of eugenol and isoeugenol on DNA synthesis and metabolic activity of HaCaT cells

In order to support previous results concerning the eugenol/isoeugenol-induced cell cycle arrest in HaCaT cells the impact on DNA synthesis as an additional parameter for cell cycle progression was investigated by using a BrdU incorporation assay. At the same time, the metabolic activity of HaCaT cells was assessed and the cell numbers were determined. Therefore, cells were incubated with varying concentrations of eugenol (60 µM, 150 µM, 300 µM, and 600 µM) or isoeugenol (60 µM, 150 µM, and 300 µM) and the solvent DMSO alone (1%) for 48 h.

As demonstrated in figures 12A and 12B, the proliferation rate of HaCaT cells was decreased by eugenol and isoeugenol at their highest concentrations. Compared to solvent treated cells, 600 µM eugenol caused a reduction of the proliferation rate to 54% (n=3, SEM=5.7, $p<0.001$). The effects were even more evident when isoeugenol was applied. 150 µM isoeugenol decreased the proliferation rate to 46% (n=3, SEM=5.7, $p<0.001$) and 300 µM isoeugenol caused a reduction to 8% (n=3, SEM=1.5, $p<0.001$). In addition, both substances also decreased the metabolic activity of the cell population (figures 12C and 12D). Compared to DMSO treated cells, 600 µM eugenol reduced the metabolic activity to 56% (n=3, SEM=1.2, $p<0.001$), whereas 300 µM isoeugenol caused a decrease to 57% (n=3, SEM=6.4, $p<0.001$). In accordance with the reduction of metabolic activity and proliferation rate in HaCaT cells, both compounds decreased the cell numbers depending on their dose (figure 13).

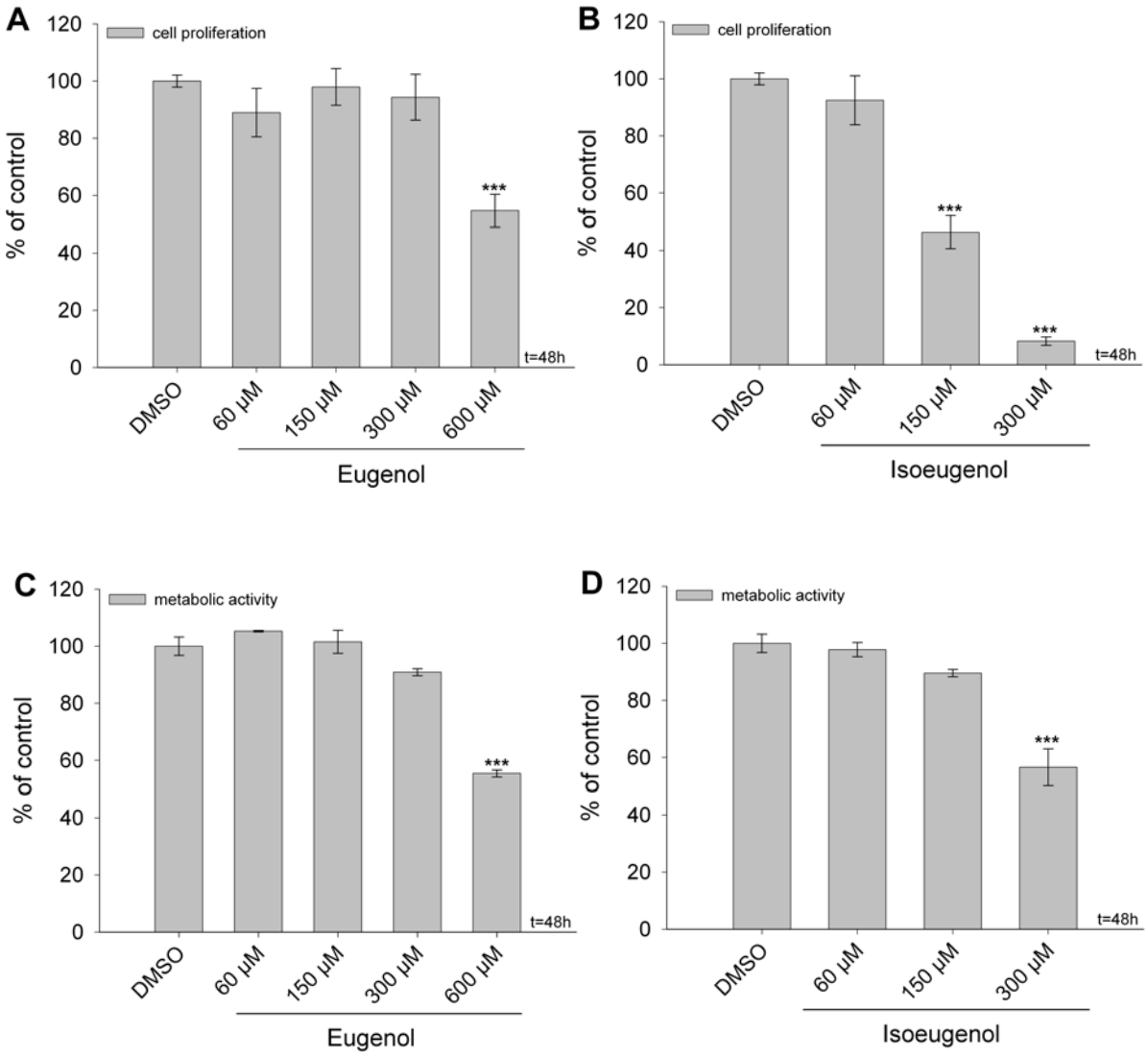


Figure 12: Effects of eugenol and isoeugenol on DNA synthesis and metabolic activity of HaCaT cells

HaCaT cells were incubated in the presence of the solvent DMSO alone (1%, negative control) and varying concentrations of either eugenol (60 µM - 600 µM) or isoeugenol (60 µM - 300 µM) for 48 h. Bars represent the mean of three independent experiments +/- SEM and asterisks indicate significant differences from DMSO treated controls (*p<0.05, **p<0.01, ***p<0.001). DNA synthesis was assessed via BrdU incorporation. (A) Incubation with eugenol. (B) Incubation with isoeugenol. Metabolic activity was determined using the WST-1 assay. (C) Incubation with eugenol. (D) Incubation with isoeugenol.

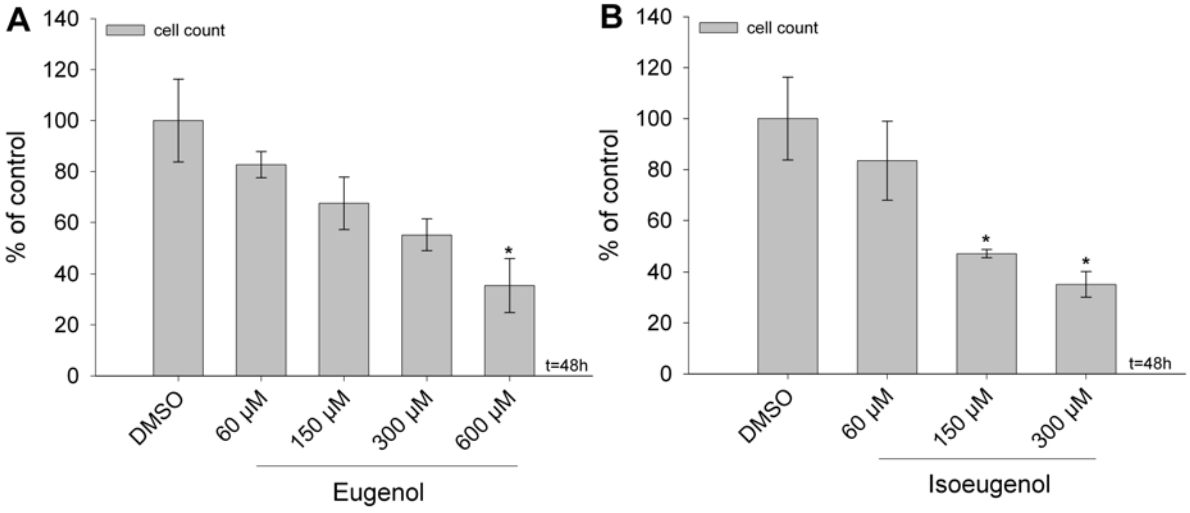


Figure 13: Effects of eugenol and isoeugenol on cell numbers of HaCaT cells

HaCaT cells were incubated in the presence of the solvent DMSO alone (1%, negative control) and varying concentrations of either eugenol (60 µM - 600 µM) or isoeugenol (60 µM - 300 µM) for 48 h. Following incubation, cell numbers were determined. Bars represent the mean of three independent experiments +/- SEM and asterisks indicate significant differences from DMSO treated controls (*p<0.05).

Very similar effects of eugenol and isoeugenol on DNA synthesis and metabolic activity were detected in siAhR HaCaT cells (figure 14). Both parameters were decreased by the two substances. Compared to control cells, 600 µM eugenol reduced the proliferation rate of siAhR HaCaT cells statistically significant to 55% (n=3, SEM=5.8, p<0.001) (figure 14A) and 300 µM isoeugenol decreased the rate to 9% (n=3, SEM=1.4, p<0.001) (figure 14B) when compared to DMSO treated cells. In consistence with the results obtained for wildtype HaCaT cells, both compounds decreased the metabolic activity of siAhR HaCaT cells, as well (figure 14C and 14D). 600 µM eugenol reduced the metabolic activity of siAhR HaCaT cells to 60% (n=3, SEM=2.4, p<0.001) (figure 14C), whereas 300 µM isoeugenol caused a decrease to 38% (n=3, SEM=4.2, p<0.001) (figure 14D). In accordance with the reduction of proliferation rate and metabolic activity in siAhR HaCaT cells, both compounds also significantly decreased the cell numbers in these cells depending on their dose (figure 15).

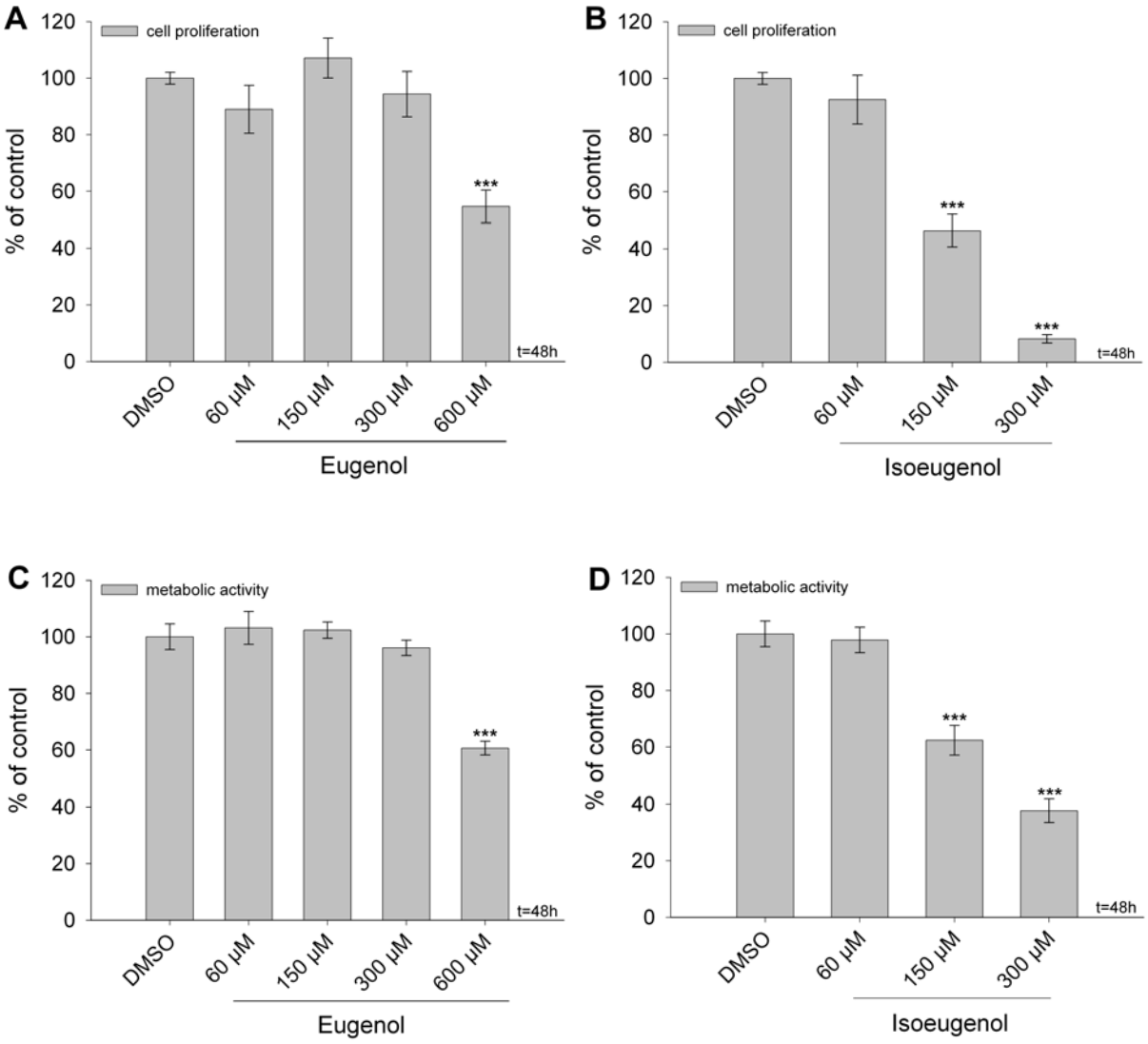


Figure 14: Effects of eugenol and isoeugenol on DNA synthesis and metabolic activity of siAhR HaCaT cells

siAhR HaCaT cells were incubated in the presence of the solvent DMSO alone (1%, negative control) and varying concentrations of either eugenol (60 µM - 600 µM) or isoeugenol (60 µM - 300 µM) for 48 h. Bars represent the mean of three independent experiments +/- SEM and asterisks indicate significant differences from DMSO treated controls (*p<0.05, **p<0.01, ***p<0.001). DNA synthesis was assessed via BrdU incorporation. (A) Incubation with eugenol. (B) Incubation with isoeugenol. Metabolic activity was determined using the WST-1 assay. (C) Incubation with eugenol. (D) Incubation with isoeugenol.

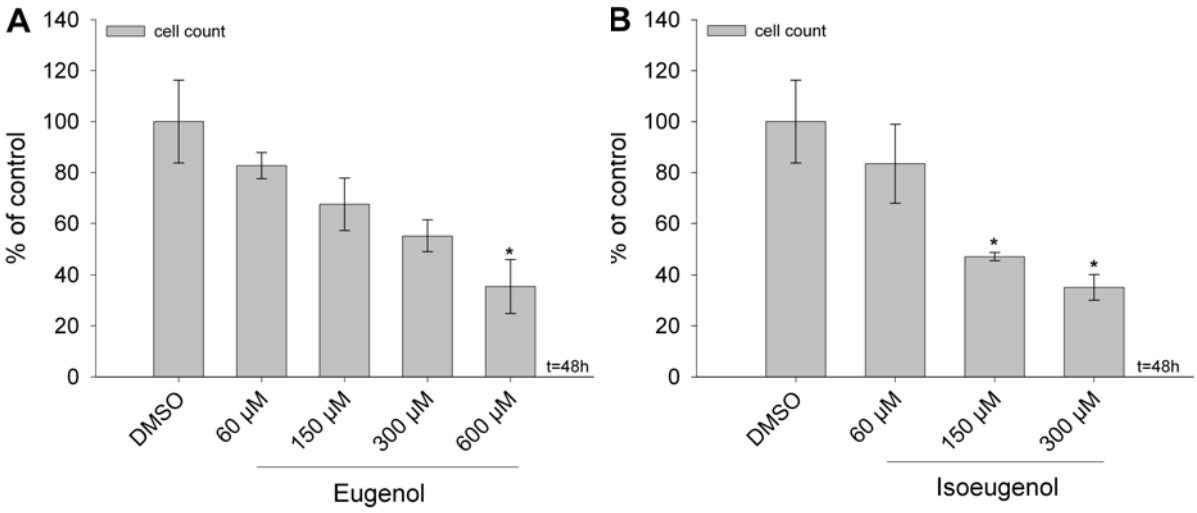


Figure 15: Effects of eugenol and isoeugenol on cell numbers of siAhR HaCaT cells

siAhR HaCaT cells were incubated in the presence of the solvent DMSO alone (1%, negative control) and varying concentrations of either eugenol (60 µM - 600 µM) or isoeugenol (60 µM - 300 µM) for 48 h. Following incubation cell numbers were determined. Bars represent the mean of three independent experiments +/- SEM and asterisks indicate significant differences from DMSO treated controls (*p<0.05).

4.4.4 Impact of eugenol and isoeugenol on cell cycle regulatory proteins

The incubation of HaCaT cells with eugenol and isoeugenol induced an AhR dependent G_0/G_1 arrest of HaCaT cell cycle. In order to reveal the underlying molecular mechanisms, the influence of eugenol and isoeugenol on cell cycle regulatory proteins involved in the transition from G_0/G_1 to S phase of the cell cycle was studied. Therefore, synchronized HaCaT cells were incubated with eugenol (600 µM) and isoeugenol (300 µM), or the solvent DMSO alone (1%) for 24 h. Subsequently, protein levels of $p27^{Kip1}$, pRB, CDK2, CDK4, and CDK6 were determined via western blot analysis. Because of its strong and unaltered protein level, ARNT was used as an additional loading control to Ponceau-S staining in several cases. Figure 16 demonstrates that both eugenol and isoeugenol increased the protein level of $p27^{Kip1}$. Reduced protein levels after eugenol or isoeugenol treatment were determined for pRB (figure 17), CDK4 (figure 19), and CDK6 (figure 20). The protein levels for CDK2 (figure 18) and ARNT remained unchanged after fragrance incubation.

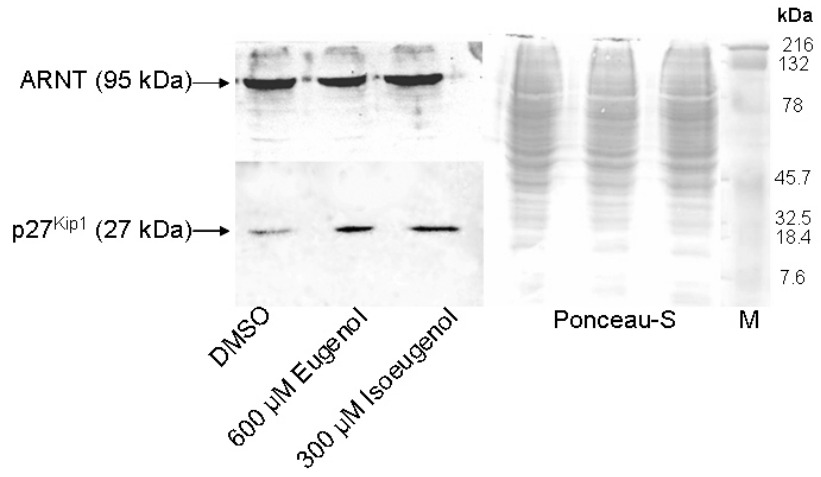


Figure 16: Impact of eugenol and isoeugenol on p27^{Kip1}protein level

Synchronized HaCaT cells were incubated with the solvent DMSO alone (1%, negative control), eugenol (600 µM), or isoeugenol (300 µM) for 24 h. Protein lysates were prepared and 100 µg total protein were subjected to SDS-PAGE followed by western blot analysis. Equal protein loading was ensured by Ponceau-S staining and ARNT served as an additional loading control. M: kaleidoscope prestained standard.

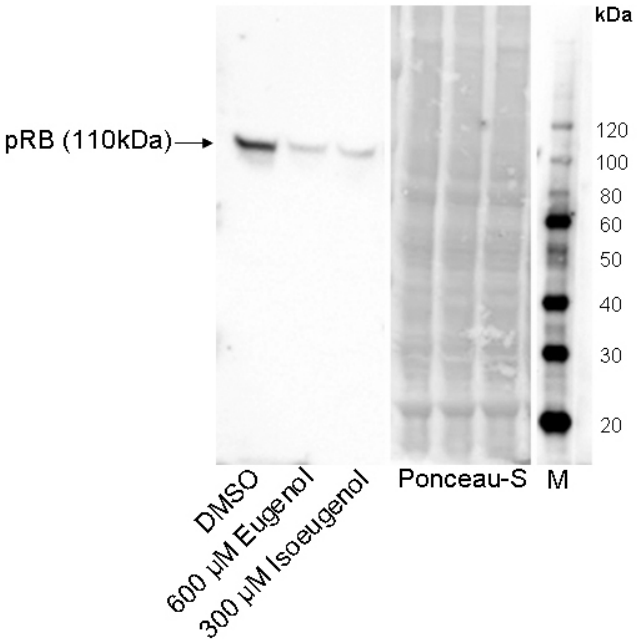


Figure 17: Impact of eugenol and isoeugenol on pRB protein level

Synchronized HaCaT cells were incubated with the solvent DMSO alone (1%, negative control), eugenol (600 µM), or isoeugenol (300 µM) for 24 h. Protein lysates were prepared and 25 µg total protein were subjected to SDS-PAGE followed by western blot analysis. Equal protein loading was ensured by Ponceau-S staining. M: MagicMark^{TM} XP.

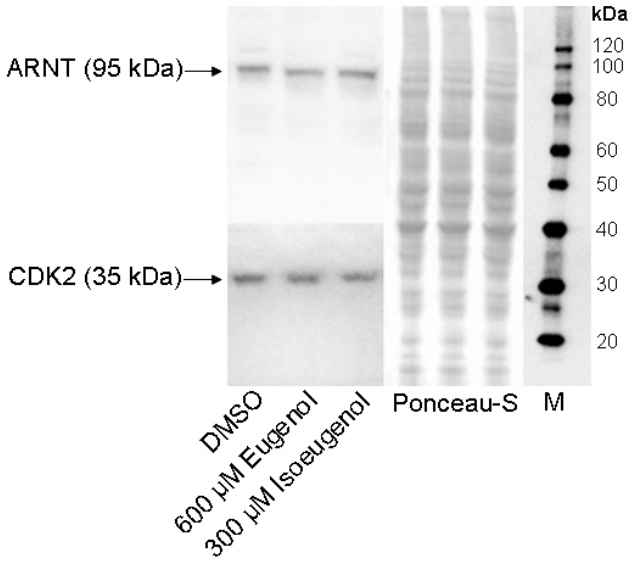


Figure 18: Impact of eugenol and isoeugenol on CDK2 protein level

Synchronized HaCaT cells were incubated with the solvent DMSO alone (1%, negative control), eugenol (600 µM), or isoeugenol (300 µM) for 24 h. Protein lysates were prepared and 25 µg total protein were subjected to SDS-PAGE followed by western blot analysis. Equal protein loading was ensured by Ponceau-S staining and ARNT served as an additional loading control. M: MagicMark™ XP.

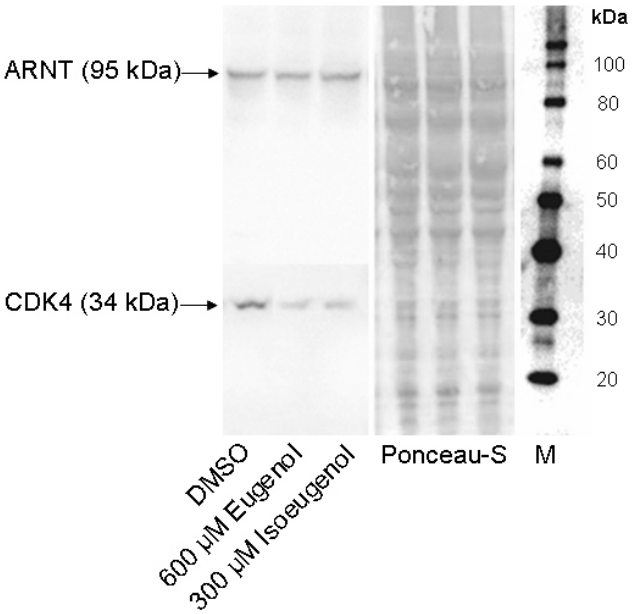


Figure 19: Impact of eugenol and isoeugenol on CDK4 protein level

Synchronized HaCaT cells were incubated with the solvent DMSO alone (1%, negative control), eugenol (600 µM), or isoeugenol (300 µM) for 24 h. Protein lysates were prepared and 25 µg total protein were subjected to SDS-PAGE followed by western blot analysis. Equal protein loading was ensured by Ponceau-S staining and ARNT served as an additional loading control. M: MagicMark™ XP.

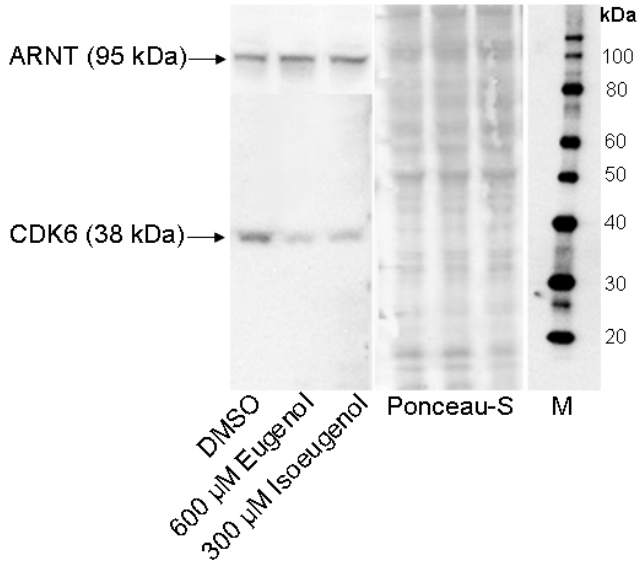


Figure 20: Impact of eugenol and isoeugenol on CDK6 protein level

Synchronized HaCaT cells were incubated with the solvent DMSO alone (1%, negative control), eugenol (600 µM), or isoeugenol (300 µM) for 24 h. Protein lysates were prepared and 25 µg total protein were subjected to SDS-PAGE followed by western blot analysis. Equal protein loading was ensured by Ponceau-S staining and ARNT served as an additional loading control. M: MagicMark™ XP.

4.5 Comparison of HaCaT and siAhR HaCaT cells

4.5.1 Cell cycle state distribution of wildtype HaCaT cells in comparison to siAhR HaCaT cells

Evidence from different studies arise for the AhR being involved in cell cycle progression of normally cycling cells in the absence of exogenous ligands. In order to proof whether this effect is also found in skin cells, cell cycle phase distribution of wildtype HaCaT cells (80% confluency) was compared to that of siAhR HaCaT cells. Figure 21 demonstrates the differences in cell cycle phase distribution between both cell lines. Wildtype HaCaT cells showed a distribution of 53% of cells in the G_0/G_1 phase (n=3, SEM=0.2), 29% of cells in the S phase (n=3, SEM=1.5), and 14% in the G_2/M phase. In the siAhR HaCaT cells the percentage of cells in the G_0/G_1 phase was significantly increased (72%, n=3, SEM=5.9, $p<0.05$) in comparison to wildtype HaCaT cells. This increase was accompanied by corresponding lower percentages of cells in the S phase (14%, n=3, SEM=2.7) and in the G_2/M phase (12%, n=3, SEM=2.5).

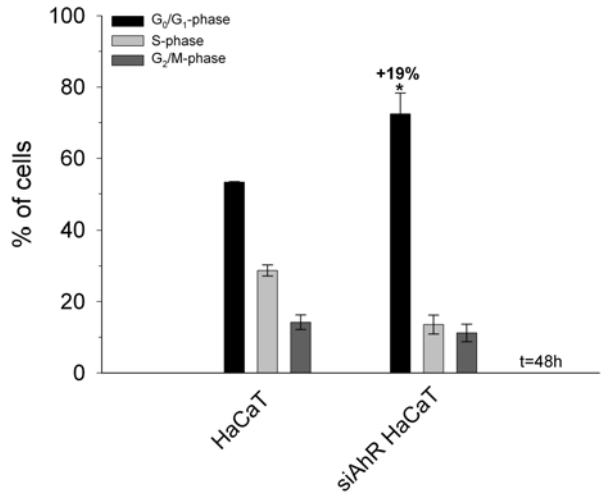


Figure 21: Comparison of cell cycle phase distribution of wildtype HaCaT and siAhR HaCaT cells

Cell cycle state distribution of wildtype HaCaT cells was characterized in comparison to siAhR HaCaT cells. The cell cycle analysis was performed after cells reached a confluency of approximately 80% via flow cytometry to determine the percentage of cells in the G_0/G_1, S, and G_2/M phases. Bars represent the mean of three independent experiments +/- SEM and asterisks indicate significant differences from wildtype HaCaT cells (*$p<0.05$).

4.5.2 DNA synthesis rate and metabolic activity of wildtype HaCaT cells compared to siAhR HaCaT cells

As presented in section 4.5.1, the percentage of cells in the G_0/G_1 phase of siAhR HaCaT cells is significantly increased compared to wildtype HaCaT cells. In order to support these results, DNA synthesis rate and metabolic activity of wildtype and siAhR HaCaT cells were estimated. Therefore, these parameters were investigated after both cell lines reached a confluency of approximately 80%. As demonstrated in figure 22A DNA synthesis rate was significantly decreased to 78% (n=3, SEM=2.3, p< 0,001) in siAhR HaCaT cells compared to wildtype HaCaT cells. Additionally, metabolic activity was reduced to 75% (n=3, SEM=3.4, p<0.001) in siAhR HaCaT cells compared to wildtype HaCaT cells (figure 22B).

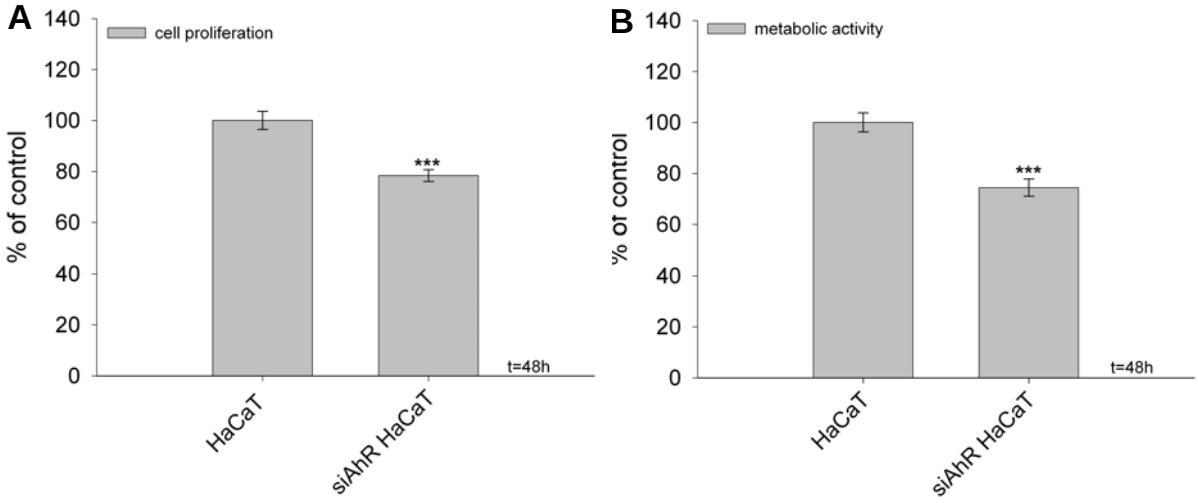


Figure 22: Comparison of DNA synthesis and metabolic activity of wildtype HaCaT and siAhR HaCaT cells

DNA synthesis rate and metabolic activity were determined for wildtype HaCaT and siAhR HaCaT cells after reaching a confluency of approximately 80%. Bars represent the mean of three independent experiments +/- SEM and asterisks indicate significant differences from wildtype HaCaT cells (***p<0.001) . (A) DNA synthesis was assessed via BrdU incorporation. (B) Metabolic activity was determined using the WST-1 assay.

4.5.3 Modulation of cell cycle regulatory proteins in wildtype HaCaT cells compared to siAhR HaCaT cells

When compared to wildtype cells, siAhR HaCaT cells exhibit a decreased proliferation rate, a reduced metabolic activity, and an altered cell cycle state distribution due to increased percentages of cells in the G_0/G_1 phase. In order to reveal the underlying molecular mechanisms of the accumulation of cells in the G_0/G_1 phase protein levels of polypeptides involved in the transition from G_0/G_1 to S phase were determined in both cell lines. Therefore, synchronized cells were cultivated until both cell lines reached a confluency of approximately 80% and protein levels of $p27^{Kip1}$, pRB, CDK2, CDK4, and CDK6 were determined via western blot analysis. The protein level of ARNT was also determined for both cell lines. As demonstrated in figure 23, an increased protein level of $p27^{Kip1}$ was detected in siAhR HaCaT cells. Protein levels of pRB (figure 24), CDK2 (figure 25), and ARNT (figure 28) were decreased in siAhR HaCaT cells, whereas protein levels of CDK4 (figure 26) and CDK6 (figure 27) remained equal in both cell lines.

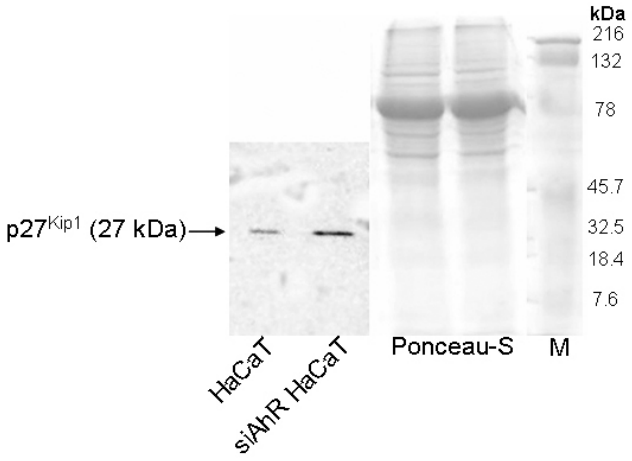


Figure 23: $p27^{Kip1}$ protein level in wildtype HaCaT compared to siAhR HaCaT cells

Cells were synchronized via serum starvation for 24 h. After serum readdition cells were cultivated until they reached a confluency of approximately 80%. Protein lysates were prepared and 25 µg of total protein were subjected to SDS-PAGE followed by western blot analysis. Equal protein loading was ensured by Ponceau-S staining. M: kaleidoscope prestained standard.

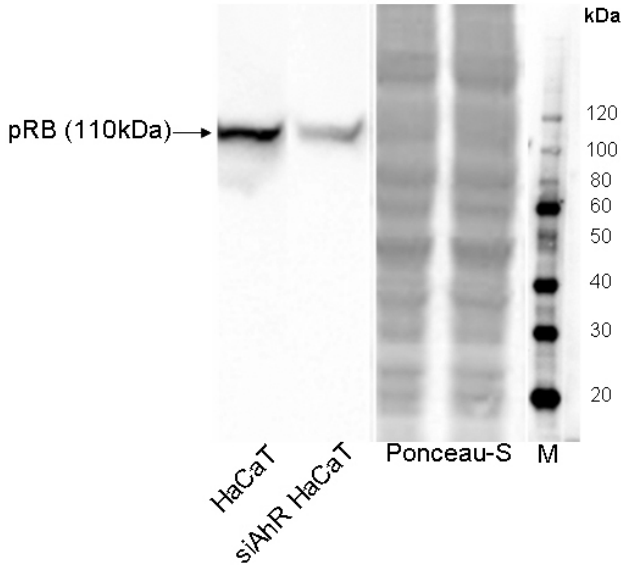


Figure 24: pRB protein level in wildtype HaCaT compared to siAhR HaCaT cells

Cells were synchronized via serum starvation for 24 h. After serum readdition cells were cultivated until they reached a confluency of approximately 80%. Protein lysates were prepared and 25 µg of total protein were subjected to SDS-PAGE followed by western blot analysis. Equal protein loading was ensured by Ponceau-S staining. M: MagicMark™ XP.

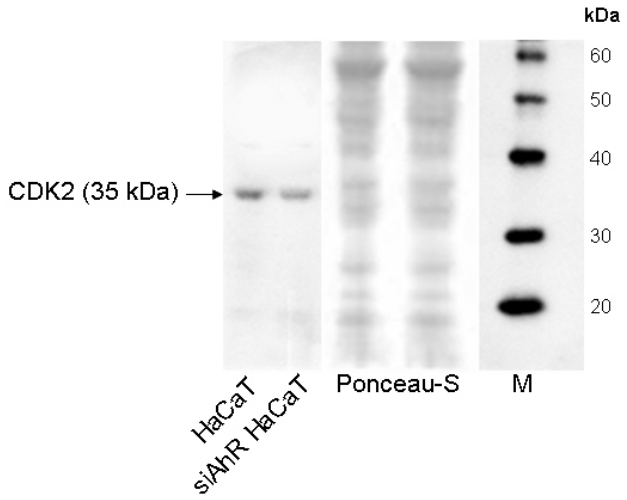


Figure 25: CDK2 protein level in wildtype HaCaT compared to siAhR HaCaT cells

Cells were synchronized via serum starvation for 24 h. After serum readdition cells were cultivated until they reached a confluency of approximately 80%. Protein lysates were prepared and 25 µg of total protein were subjected to SDS-PAGE followed by western blot analysis. Equal protein loading was ensured by Ponceau-S staining. M: MagicMark™ XP.

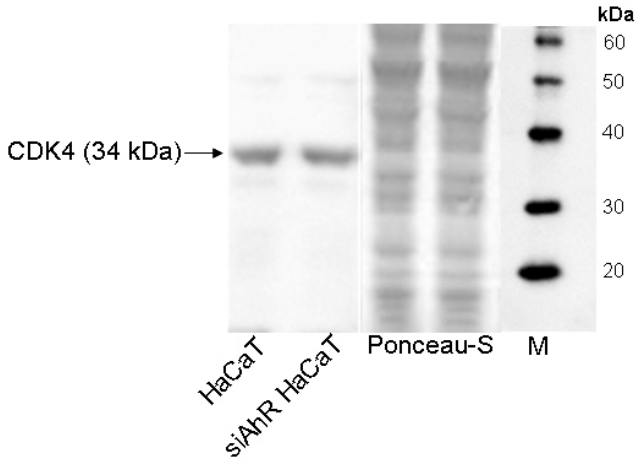


Figure 26: CDK4 protein level in wildtype HaCaT compared to siAhR HaCaT cells

Cells were synchronized via serum starvation for 24 h. After serum readdition cells were cultivated until they reached a confluency of approximately 80%. Protein lysates were prepared and 25 µg of total protein were subjected to SDS-PAGE followed by western blot analysis. Equal protein loading was ensured by Ponceau-S staining. M: MagicMark^{TM} XP.

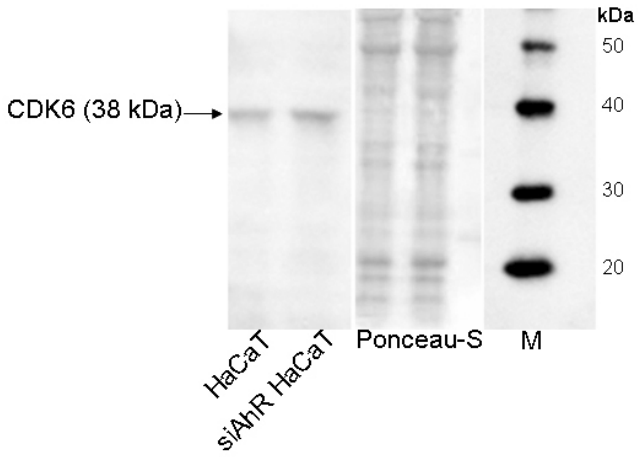


Figure 27: CDK6 protein level in wildtype HaCaT compared to siAhR HaCaT cells

Cells were synchronized via serum starvation for 24 h. After serum readdition cells were cultivated until they reached a confluency of approximately 80%. Protein lysates were prepared and 25 µg of total protein were subjected to SDS-PAGE followed by western blot analysis. Equal protein loading was ensured by Ponceau-S staining. M: MagicMark^{TM} XP.

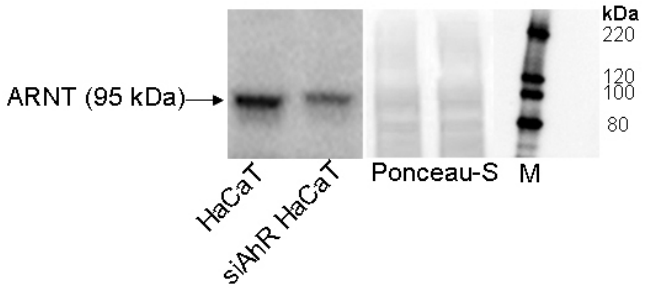


Figure 28: ARNT protein level in wildtype HaCaT compared to siAhR HaCaT cells

Cells were synchronized via serum starvation for 24 h. After serum readdition cells were cultivated until they reached a confluency of approximately 80%. Protein lysates were prepared and 25 µg of total protein were subjected to SDS-PAGE followed by western blot analysis. Equal protein loading was ensured by Ponceau-S staining. M: MagicMark^{TM} XP.

5. Discussion

The exposure of human skin to synthetic exogenous compounds has been associated with a number of outcomes. The most widely recognized effects on human skin from exposure to dioxin and dioxin-like substances for example are chloracne, skin rashes, hyperkeratinisation, and hyperpigmentation (Greene et al., 2003; Schecter et al., 2006). Such kind of exposures are worst-case-scenarios and actually more relevant is how frequently used substances behave when human skin is exposed to them. Once absorbed, these substances may interact with different biomolecules in skin, leading to various physiological responses. Depending on the substance, the AhR signalling pathway is one possible mechanism whereby the skin responds to exposures, predominantly through the induction of metabolizing enzymes. Moreover, one known physiological function of the AhR in many tissues is its impact on the control of cell cycle progression (cf. section 1.2.5). Although its role in metabolism is well-characterized in skin, very little is known about the physiological function of the AhR in this tissue in the absence of exogenous ligands. Within this thesis, the impact of naturally occurring exogenous substances on the AhR signalling pathway in skin cells was studied for the phenolic derivatives eugenol and isoeugenol. These two substances which are due to their odour referred to as fragrances are ubiquitous in our daily lives (cf. section 1.1). This ubiquitous distribution results in an unavoidable exposure of skin to such substances. Eugenol and isoeugenol were used to investigate the potential influence on the AhR because of their known antiproliferative properties and the established function for eugenol in inducing the expression of *CYP1A1*, one of the best characterized AhR target genes (Al Masaoudi et al., 2001). The inducible AhR dependent expression of *CYP1A1* (Ledirac et al., 1997), as well as the AhR signalling pathway related to metabolism (Henley et al., 2004; Ikuta et al., 2004) are well-documented in the human immortalized keratinocyte cell line HaCaT (Boukamp et al., 1988), what makes this cell line appropriate for studies on AhR signalling. This cell line is moreover suitable for studies on skin (Dvorak et al., 2006) and was therefore adopted as a model system within this thesis. Keratinocytes are the most appropriate cells for studying effects of skin exposure. The epidermis which is the outermost layer of skin, is the major site of direct exposure and predominantly consists of keratinocytes. An AhR knockdown variant of this cell line was also a suitable tool to study the physiological function of the AhR in cell cycle control in the absence of exogenous ligands.

5.1 Impact of eugenol and isoeugenol on the intracellular localization of the AhR and on AhR target genes in HaCaT cells

The ubiquitous distribution of eugenol and isoeugenol results in an almost unavoidable contact with these substances in our daily lives. Despite this fact, their molecular mechanisms of action in skin are poorly understood. As described above, previous investigations of our working group showed that eugenol induced *CYP1A1* mRNA in primary human keratinocytes (Al Masaoudi et al., 2001). Knowing that *CYP1A1* expression is modulated by the AhR, the impact of eugenol and isoeugenol on the localization of the AhR in HaCaT cells was studied. In its unliganded form, the AhR is present in the cytosolic compartment (cf. section 1.2.2). Upon ligand binding, the AhR translocates into the nucleus. Activation of the AhR by exogenous ligands is thus detectable through the intracellular distribution of the receptor.

In consistence with results from Ikuta and colleagues (Ikuta et al., 2004) it could be shown that under normal cell culture conditions and a confluency of 80-90% the AhR is diffusely distributed predominantly in the cytoplasm of HaCaT cells. Treatment of HaCaT cells with DMSO alone did not modify the distribution of the AhR. Additional treatment of HaCaT cells with either eugenol or isoeugenol resulted in a rapidly and evident translocation of the AhR from the cytoplasm into the nucleus. This effect was detectable 90 min after incubation. The eugenol/isoeugenol-induced translocation is comparable to that induced by the positive control benzo[a]pyrene (B[a]P), a known ligand of the AhR (Nguyen and Bradfield, 2008). Nuclear translocation of the AhR after treatment with several exogenous compounds is a well-known consequence of AhR/ligand interactions (Elbi et al., 2002). As far as we know the impact of eugenol and isoeugenol on the AhR signalling pathway was shown for the first time within this thesis and moreover, the nuclear translocation of the AhR after treatment with exogenous substances in HaCaT cells has not been examined before. Whether the translocation of the AhR was caused through a direct interaction between eugenol or isoeugenol and the AhR is not clear. It should be taken into account that the eugenol/isoeugenol-induced translocation of the AhR may be a result of indirect interactions. This provides the ability that additional pathways and the recruitment of further biomolecules play a role. As a result, it cannot be excluded that eugenol and isoeugenol are metabolized to AhR ligands (Thompson et al., 1989) or cause the release of endogenous intracellular ligands.

Nevertheless, the direct interaction between these two molecules and the AhR is of course possible. Besides the well-known classical AhR ligands like TCDD, there are a plenty of structurally diverse so-called non-classical ligands (Denison and Nagy, 2003). The naturally occurring substance curcumin for instance, a phenolic derivative in the spice

turmeric, induced the translocation of the receptor in oral squamous cell carcinoma cells and oral mucosa through direct interactions with the AhR. Electromobility shift assays demonstrated that similar to TCDD, curcumin inclusion resulted in AhR nuclear translocation and formation of the transcriptionally active AhR/ARNT complex (Rinaldi et al., 2002). The best characterized high affinity AhR ligands are anyhow planar hydrophobic molecules with electron rich regions (cf. section 1.2.3). Nevertheless, absolute planarity is not a requirement for AhR binding (McKinney and Singh, 1981). Both, eugenol and isoeugenol are hydrophobic and electron rich molecules (figure 1). They are phenolic derivatives and contain one planar aromatic ring. Due to their structural characteristics, eugenol and isoeugenol are able to adopt an almost planar structure depending on the chemical properties of their environment. Stabilized in the ligand binding pocket of the AhR for instance, a planar arrangement of these two substances might be possible. Therefore, at most four hydrogen atoms would not be in the plane of the aromatic ring and for sterical purposes, these hydrogen atoms would not require large space. A possible almost planar conformation of eugenol and isoeugenol is demonstrated in figure 29.

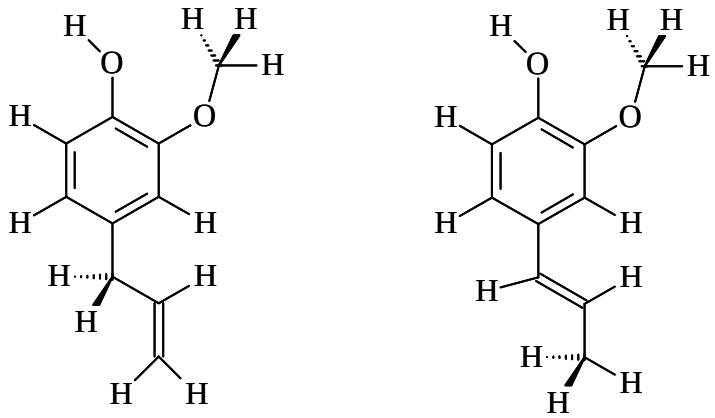


Figure 29: Possible steric conformation of eugenol and isoeugenol

Depending on their chemical environment, eugenol and isoeugenol are able to adopt an almost planar structure. Interrupted and bold type bondings indicate hydrogen atoms not lying in the plane of the aromatic ring.

Another substance also lacking the conjugated aromatic rings of PAHs and also bearing little structural resemblance to classical AhR ligands is phenylthiourea (Nguyen and Bradfield, 2008) (figure 4). This almost planar molecule is reported to activate the AhR and bears most structural resemblance to eugenol and isoeugenol regarding to all known agonists. Phenylthiourea belongs to the group of weak AhR activators requiring doses in the

mid to high micromolar range to elicit receptor activity (Nguyen and Bradfield, 2008), a characteristic also applying to eugenol and isoeugenol. Hence, it might be possible that both eugenol and isoeugenol, due to their chemical properties and their dimensions, are potential AhR ligands. This hypothesis is supported by the fact that both eugenol and isoeugenol are plant-derived derivatives and it is known that for example herbal extracts (Jeuken et al., 2003) contain AhR agonists and/or antagonists. Furthermore, plant-derived substances like red clover isoflavones (Medjakovic and Jungbauer, 2008) and indirubins (Knockaert et al., 2004) are also potent ligands of the AhR. The translocation of the AhR caused by eugenol and isoeugenol was in addition detectable 90 min after substance incubation. Translocation of the AhR caused by known ligands like indirubins was also detected at the same time (Knockaert et al., 2004). This analogy additionally emphasises the hypothesis that eugenol and isoeugenol are AhR ligands. It is known that the translocation of the AhR into the nucleus results in heterodimerization of the AhR with ARNT. Association of AhR/ARNT converts the complex into its high affinity DNA binding form whereupon it binds to XREs in order to regulate transcription of several target genes (cf. section 1.2.2). Another fact supporting the eugenol/isoeugenol-induced translocation of the AhR and thereby supporting their interaction with the receptor is the induction of AhR target genes in HaCaT cells by both substances. In consistence with the above mentioned previous investigations of our working group in primary keratinocytes, eugenol was able to induce the expression of *CYP1A1* mRNA in HaCaT cells. In contrast to that study, these effects were here also detectable for isoeugenol. Eugenol as well as isoeugenol also induced mRNA levels of the *AhRR* in HaCaT cells, a further target gene of the AhR. Altogether these results indicate that both substances might be ligands of the AhR. Nevertheless, it should be kept in mind that some substances like carbaryl which is able to induce *CYP1A1* gene expression in HepG2 and HaCaT cells, do not directly interact with the AhR. A partial AhR antagonist was indeed able to suppress carbaryl induced *CYP1A1* expression, but competitive binding studies indicated that carbaryl did not displace TCDD from the AhR (Ledirac et al., 1997).

Assuming that eugenol and isoeugenol are AhR ligands, it still remains unclear what kind of intrinsic activity both molecules may exhibit. TCDD and B[a]P for instance are pure agonists of the AhR, exhibiting strong activity via the receptor. In contrast, there also exist pure antagonists like resveratrol, not exhibiting any activity via the receptor (Singh et al., 2000). Partial antagonists like alpha-naphtoflavone are reported to moderately activate the AhR in some tissues (Ledirac et al., 1997). The efficacy of AhR ligands can moreover be tissue specific. However, consistent with AhR agonists, both eugenol and isoeugenol induced the translocation of the AhR into the nucleus and induced AhR target genes. In comparison to classical AhR agonists like benzo[a]pyrene B[a]P the eugenol/isoeugenol-induced AhR target gene induction was only moderate. In HaCaT cells

eugenol induced *CYP1A1* mRNA levels by 7 fold and isoeugenol by 4 fold, whereas B[a]P induced the levels by 166 fold. For *AhRR* eugenol induced the mRNA levels by 6 fold and isoeugenol by 4 fold. In contrast, B[a]P induced *AhRR* mRNA levels by 30 fold. Supposing that both substances indeed bind to the AhR, these results indicate that eugenol and isoeugenol are most likely partial antagonists. The well-known AhR antagonist 3´-methoxy-4´-nitroflavone (MNF) for instance, is reported to moderately activate the AhR in some tissues and thereby marginally enhance *CYP1A1* transcription (Zhou and Gasiewicz, 2003). Curcumin is like eugenol and isoeugenol a potent antioxidant and presumably a chemopreventive substance. In MCF-7 human breast cancer cells, curcumin induced *CYP1A1* expression and activity, after binding to the AhR and activating its DNA-binding capacity for the XRE of *CYP1A1*. In spite of all this, curcumin partially inhibited AhR activation by 7,12-dimethylbenz[a]antracene (DMBA) and completely inhibited DMBA-induced CYP1A1 activity in these cells (Ciolino et al., 1998). In human oral squamous cell carcinoma cells (SCC) curcumin initiated nuclear translocation of the AhR and increased expression and activity of CYP1A1. Additionally, curcumin inhibited bioactivation of benzo[a]pyrene-7R-trans-7,8-dihydrodiol in SCC cells and oral mucosal tissues (Rinaldi et al., 2002). Eugenol was moreover shown to inhibit DMBA-induced genotoxicity by inhibiting DMBA-induced *CYP1A1* induction and activity of both CYP1A1 and CYP1B1 in MCF-7 cells (Han et al., 2007). These studies additionally provide evidence for the potential partial antagonistic activity of eugenol. Due to the fact that both substances induced the translocation of the AhR into the nucleus and increased AhR target gene expression, one possible consequence of these events might be the perturbation of cell cycle progression. In many cell types the AhR seems to be distracted from its physiological role in promoting cell cycle progression after incubation with exogenous substances. Furthermore, eugenol and isoeugenol are known to have antiproliferative properties, but the underlying molecular mechanisms of these properties are not known so far.

5.2 Cell cycle progression of HaCaT cells

5.2.1 Influence of eugenol and isoeugenol on cell cycle progression of HaCaT cells

One known physiological role of the AhR in many tissues is its impact on the control of cell cycle progression (cf. section 1.2.5). In recent years, antiproliferative effects of eugenol have been detected (Ghosh et al., 2005). Due to the fact that the underlying molecular mechanisms are poorly understood, this knowledge was extended within the scope of this thesis by studying the interrelationship between eugenol and isoeugenol, their antiproliferative properties, and the AhR signalling pathway. The obtained data suggest that

eugenol and isoeugenol are able to modify cell cycle state distribution in HaCaT cells. Both substances caused an accumulation of cells in the G_0/G_1 phase of the cell cycle in normally cycling HaCaT cells, as well as in HaCaT cells which were synchronized before substances were applied. In contrast to this study, Ghosh and colleagues determined an antiproliferative effect for eugenol but not for isoeugenol due to an arrest of melanoma cells in the S phase of the cell cycle (Ghosh et al., 2005). These discrepancies may result from differences in the used eugenol/isoeugenol concentrations, or may point to cell-specific differences. Cell specific differences were also detected by Pisano and co-workers. They studied the antiproliferative effects of eugenol and eugenol-derivatives on nine different malignant melanoma cell lines which also differ from that used by Ghosh and colleagues. None of the tested melanoma cell lines exhibited an altered cell cycle progression after treatment with eugenol or isoeugenol. Only eugenol-related biphenyls exhibit antiproliferative effects on these cell lines (Pisano et al., 2007). The eugenol/isoeugenol-induced cell cycle arrest in HaCaT cells was more evident in synchronized cells when compared to normally cycling cells. Synchronized HaCaT cells were accumulated in the G_0/G_1 phase of the cell cycle before substances were added. On the background that normal cell cycle of HaCaT cells requires approximately 24 h, it seems that a fraction of the cells directly remained in the G_0/G_1 phase of the cell cycle due to the application of eugenol and isoeugenol. In contrast, normally cycling cells which have already passed the restriction point of the cell cycle before eugenol and isoeugenol were added, apparently finished this cycle completely, although both substances were present. When entering a new cycle, cells seemed to be directly arrested in the G_0/G_1 phase due to the presence of eugenol and isoeugenol, explaining the time delay of the effect compared to synchronized cells. The increased percentage of cells in the G_0/G_1 phase after substance incubation was accompanied by corresponding lower percentages of cells in the S and G_2/M phases of the cell cycle, compared to solvent treated cells. As a consequence of the eugenol/isoeugenol-induced cell cycle arrest, the DNA synthesis rate as a parameter for proliferation of HaCaT cells was also inhibited. Moreover, cell numbers and overall metabolic activity were reduced. Due to the fact that only non-toxic concentrations were applied and the subG_0 phase, as a parameter for late apoptosis or necrosis was not enhanced (data not shown), apoptosis or necrosis can be excluded as a reason for the reduced cell numbers. Rather, the reduced cell numbers result from the eugenol/isoeugenol-induced cell cycle arrest and inhibited proliferation. The observed effects were more evident in the presence of isoeugenol when compared to eugenol. These differences may result from distinctions in the chemical properties of eugenol and isoeugenol, their possible interaction with different biomolecules, and also from differences in metabolism of the two isomers (Bertrand et al., 1997).

To summarize, in consistence with the literature eugenol and isoeugenol exhibit antiproliferative properties. The underlying molecular mechanisms are not clear so far and therefore, the influence of the AhR on cell cycle progression of HaCaT cells was studied. Moreover, it was determined whether the AhR impacts on the eugenol/isoeugenol-induced cell cycle arrest.

5.2.2 Role of the AhR in cell cycle progression of HaCaT cells

The AhR was discovered as the receptor for dioxin and other related man-made pollutants in the 1970s. A phylogenetic survey indicates that the AhR arose over 450 million years ago, thus long before environmental pollution by anthropogenic compounds started (Hahn, 2002). Moreover, the AhR is expressed in various tissues and at different times during development, being inconsistent with a unique role in defense against environmental chemicals (Jain et al., 1998; Kuchenhoff et al., 1999). By extending research in AhR biology, evidence appeared that the AhR interacts with endogenous ligands and generally plays an important role in cell physiology. Loss of function studies in mice suggested that in the absence of exogenous ligands the AhR plays a role in growth (Schmidt et al., 1996) and development (Fernandez-Salguero et al., 1995; Lahvis et al., 2000). In skin, up to now little is known about the physiological function of the AhR. In order to expand this knowledge, involvement of the AhR in cell cycle progression of HaCaT cells was studied in the absence of exogenous ligands. An AhR knockdown variant of HaCaT cells (siAhR HaCaT) was used to address this subject. The protein level of the AhR in this cell line is reduced by 80% (Fritsche et al., 2007). It is known from literature that disruption of AhR expression with antisense oligonucleotides or siRNA results in decreased mouse blastocyst development (Peters and Wiley, 1995) and abnormalities in cell cycle regulation. Evidence has grown that the absence or downregulation of the AhR results in prolongation of the cell cycle. Within the scope of this thesis this knowledge was expanded on keratinocytes. The investigated siAhR HaCaT cells exhibited changes in cell cycle state distribution in comparison to wildtype HaCaT cells. Significantly more cells were detected in the G_0/G_1 phase of the cell cycle in siAhR HaCaT cells than in wildtype HaCaT cells. As a consequence of these results, cell proliferation and metabolic activity of siAhR HaCaT cells were decreased in comparison to wildtype HaCaT cells. So far, similar results were obtained for AhR-negative mouse hepatoma Hepa1c1c7 cell variants (Ma and Whitlock, 1996) and human HepG2 hepatoma cells transfected with a siRNA targeted against the AhR (Abdelrahim et al., 2003). Both cell lines exhibit a slower progression through the cell cycle than respective wildtype cells. In consistence with results gained within this thesis, the inhibition of cell cycle progression in both cell variants was caused by a delay in the transition from G_1 to S phase of the cell cycle. In Hepa 1c1c7 cells this effect was reversed by ectopic expression of the AhR (Ma and Whitlock, 1996). AhR

negative murine fibroblasts proliferated significantly slower than AhR positive fibroblasts due to an elongation of the G_1 and S phases of the cell cycle. Gene expression profiling identified a group of proteins regulating the G_1/S transition including cyclins A2, B1 and E1 which were downregulated by the lack of AhR expression. Neither exposure to a prototypical ligand nor deletion of the ligand binding domain changed their proliferation rate (Chang et al., 2007). These results confirm that at least in some tissues, the AhR indeed promote cell cylce progression.

Not every cell cycle perturbation after modifying AhR expression is caused by an altered G_1 to S transition. Cell type dependencies were found by studying mouse embryonic fibroblasts (MEFs) from AhR null mice which exhibit slower proliferation rates than cells of wildtype mice (Tohkin et al., 2000) due to an accumulation of cells in the G_2/M phase of the cell cycle (Elizondo et al., 2000). The obtained results confirm that the AhR generally plays a role in the promotion of cell cycle progression and that this role is independent of exogenous ligands. Moreover, downregulation of the AhR is combined with impaired tumorigenicity in a subcutaneous mouse xenograft model (Mulero-Navarro et al., 2005). In contrast, siRNA-dependent decreases in AhR protein in MCF-7 human breast cancer cells were accompanied by increased G_0/G_1 to S transition, suggesting an growth-inhibitory physiological role for the AhR (Abdelrahim et al., 2003). Hence, depending on the cell context the AhR exhibits growth-inhibitory or growth-promoting activity in the absence of exogenous ligands. The requirement of a functional AhR for normal cell cycle progression was further supported by overexpression studies, also obtaining contradictive results. Cells of the human T cell line Jurkat, expressing a constitutive active form of the AhR, exhibit inhibited cell growth caused by an accumulation of cells in the G_1 phase of the cell cycle (Ito et al., 2004). This may be explained by the fact that modulation of AhR activity is among others controlled by CYP1A1 through regulating the level(s) of (a) physiological receptor ligand(s) (Levine-Fridman et al., 2004). In this context, growth factor stimulated *CYP1A1* induction during G_1 to S transition likely inactivates the AhR by rapidly depleting of endogenous ligands, thereby promoting cell cycle progression. Contradictive results were obtained for clones derived from human lung epithelial A549 cells overexpressing the AhR. These cells exhibit accelerated growth and increased DNA synthesis activity compared to wildtype cells (Shimba et al., 2002). It is moreover interesting that AhR concentrations appeared to be substantially higher in many cancer cells like human pancreatic cancer cells (Koliopanos et al., 2002) and human lung carcinoma cells (Lin et al., 2003) compared to corresponding normal cells. In pancreatic cancer cells with high AhR expression levels, TCDD and diverse other relatively non-toxic AhR agonists caused growth inhibition via cell cycle arrest (Koliopanos et al., 2002). These relatively non-toxic AhR ligands may play a role in the therapy of cancer and suggestions arouse that the AhR functions as a target for the

development of chemotherapeutic ligands for several types of cancer (Bradshaw et al., 2002), including hormone-dependent cancers (Safe and McDougal, 2002) and breast cancer (Loaiza-Perez et al., 2004). As a result, relatively non-toxic substances activating the AhR signalling pathway like eugenol and isoeugenol therefore may have a potential role in the development of novel therapeutic agents for proliferative diseases such as cancer. Arguments contradictive to the latter assumption suggest that the AhR may function as a tumour suppressor gene that becomes silenced in the process of tumour formation and cancer progression (Peng et al., 2008). However, these conflicting results demonstrate that the exact molecular mechanisms of the AhR in cell cycle modulation are very poorly understood and definitely need further investigations.

5.2.3 Impact of the AhR on the eugenol/isoeugenol-induced cell cycle arrest in HaCaT cells

In the absence of exogenous ligands, the AhR promotes cell cycle progression in HaCaT cells and several other cell types (cf. 5.2.2). In the presence of ascertain exogenous ligands, the AhR seems to be distracted from this physiological function and induces cell cycle arrests in many circumstances, depending on the studied cells. In order to investigate the molecular mechanisms of the eugenol/isoeugenol-induced cell cycle arrest, a possible influence of the AhR on this cell cycle arrest was studied. The flavonoid 3´-methoxy-4´-nitroflavone (MNF) is a well-known AhR antagonist (Lu et al., 1995; Gasiewicz et al., 1996). In 5L rat hepatoma cells TCDD arrested the cells in the G_1 phase of the cell cycle in an AhR dependent manner and this arrest was completely abolished by co-treatment of TCDD with MNF (Elferink, 2003). In human oral keratinocytes MNF blocked the effects of TCDD on *CYP1A1* expression (Zhang et al., 2007). Co-incubation of HaCaT cells with eugenol or isoeugenol in the presence of MNF should therefore provide evidence whether the eugenol/isoeugenol-induced cell cycle arrest is dependent on the AhR. Similar to the effects observed for TCDD, eugenol completely lost its ability to accumulate HaCaT cells in the G_0/G_1 phase of the cell cycle in the presence of MNF. In contrast, the isoeugenol-induced cell cycle arrest was prevented only by a minor extent. Nevertheless, it was shown for the first time that the antiproliferative properties of eugenol and isoeugenol are at least in part AhR dependent. The different behaviour of eugenol and isoeugenol in the presence of MNF may have several reasons. As discussed in section 5.1, it remains to be studied whether eugenol and isoeugenol directly interact with the AhR. Assuming that indeed both substances interact directly, isoeugenol seems to have a higher affinity to the AhR than eugenol. As a consequence, eugenol would be displaced more easily from the AhR by antagonists than isoeugenol, explaining their different behaviours in co-treatment experiments with MNF. This might also explain the higher extent of the isoeugenol-induced cell cycle arrest and the

stronger inhibition of cell proliferation, despite of lower isoeugenol concentrations (cf. section 5.2.1). It is moreover possible that these differences again may result from distinctions in the chemical properties of eugenol and isoeugenol, their possible interaction with different biomolecules, and also from differences in metabolism of the two isomers (Bertrand et al., 1997). In order to further confirm the AhR dependence on the eugenol/isoeugenol-induced cell cycle arrest, AhR knockdown cells were analyzed after substance incubation. Interestingly, the eugenol/isoeugenol-induced cell cycle arrest was even more evident in this variant of HaCaT cells than in wildtype HaCaT cells (both without synchronization). Similar to wildtype HaCaT cells (cf. section 5.2.1), isoeugenol caused the effect to a higher extend than eugenol. In siAhR HaCaT cells MNF was able to prevent the eugenol-induced cell cycle arrest, whereas the isoeugenol-induced arrest was abolished to a minor extent. As described in section 5.2.2, siAhR HaCaT cells exhibit a prolonged transition from the G_0/G_1 phase of the cell cycle to the S phase due to the minor level of AhR protein. Nevertheless, the residual 20% of the receptor were presumably sufficient to interact with eugenol and isoeugenol and to cause an additional accumulation of cells in the G_0/G_1 phase of the cell cycle. In consistence with the altered cell cycle state distribution, the DNA synthesis rate was decreased in siAhR HaCaT cells after incubation with either eugenol or isoeugenol, as well as cell numbers and metabolic activity. To conclude, these results additionally underline the impact of the AhR on the eugenol/isoeugenol-induced cell cycle arrest in HaCaT cells. The effects on cell cycle progression caused by eugenol and isoeugenol are similar to those described for classical AhR ligands. Perturbation of cell cycle progression through the ligand-activated AhR is known for many circumstances. Ligand-dependent activation of the AhR signalling pathway produces varying and often contradictory effects on cell proliferation both *in vivo* and *in vitro*. These effects are species-, tissue- and cell type-dependent. Studies in skin are so far very limited. In primary human keratinocytes for instance, TCDD accelerates differentiation, inhibits senescence, and increases proliferation in an AhR dependent manner (Ray and Swanson, 2003; Ray and Swanson, 2004), while in normal human dermal fibroblasts TCDD protects the cells from apoptosis and does not enhance proliferation, rather accumulates the cells in the G_0/G_1 phase of the cell cylce (Akintobi et al., 2007). Andrysík and colleagues showed that certain PAHs enhanced cell proliferation in rat liver epithelial cells in an AhR dependent manner (Andrysik et al., 2007). However, more commonly and in consistence with results obtained within this thesis, activation of the AhR by exogenous substances mostly results in prolonged inhibition of cell proliferation and this phenomenon was observed for a variety of cell types. Studies on rodent cells for instance exhibited that B[a]P suppressed cell proliferation in Swiss mouse 3T3 cells in an AhR dependent manner (Vaziri and Faller, 1997). In mouse hepatocyte-derived cell lines TCDD induced an cell cycle arrest in the G_0/G_1 phase depending on the AhR (Mitchell et al., 2006).

Furthermore, in 5L rat hepatoma cell cultures, TCDD treatment leads to an AhR dependent delay in G_1 to S phase progression (Gottlicher et al., 1990; Weiss et al., 1996). Studies on human cell lines detected that TCDD induced an AhR dependent G_1 arrest of the cell cycle, for example in SK-N-SH human neuronal cells. In line with these results, many of the non-classical AhR ligands are known to induce cell cycle arrests in a cell type specific manner. These known effects additionally support the assumption that both eugenol and isoeugenol might be AhR ligands. Treatment of rat 5L hepatoma cells with several different flavonoids (Reiners et al., 1999) and indirubins (Knockaert et al., 2004), resulted in an AhR dependent G_1 arrest of the cell cycle. There are moreover AhR antagonists also known to induce cell cycle arrests. 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole for instance was able to induce an AhR dependent cell cycle arrest in MCF-7 cells (Trapani et al., 2003). These observations generally indicate that the activated AhR slows down cell proliferation, supporting its potential role as a tumour suppressor (Peng et al., 2008).

In conclusion, the AhR has a pro-proliferative function in HaCaT keratinocytes as demonstrated by AhR knockdown cells and seems to loose this function due to its activation by eugenol or isoeugenol. However, the exact mechanisms controlling these effects remain to be studied.

5.2.4 AhR dependent modulation of cell cycle regulatory proteins in HaCaT cells

In the presence of eugenol and isoeugenol cell cycle progression of HaCaT cells was inhibited in an AhR dependent manner. In HaCaT cells with a reduced AhR protein level, cell proliferation was inhibited as well, pointing to a role of the AhR in promoting cell cycle progression in wildtype HaCaT cells in the absence of exogenous ligands. Cell cycle perturbation of HaCaT cells caused by either eugenol or isoeugenol or by a decreased AhR protein level was manifested through an accumulation of cells in the G_0/G_1 phase of the cell cycle. In order to reveal the exact underlying molecular mechanisms of these effects, the involvement of several cell cycle regulatory proteins was investigated. The key proteins involved in the transition from the G_1 phase of the cell cycle to the S phase are members of the pRB and E2F families of proteins (Nevins, 1998; Cobrinik, 2005), as well as the cyclin dependent kinases (CDKs) 2, 4, and 6, their corresponding cyclins, and the cyclin dependent kinase inhibitors (CKIs) (Weinberg, 1995). Two classes of CKIs exist: the first is that of the p21 or CIP/KIP family consisting of $p21^{CIP}$, $p27^{KIP1}$, and $p57^{KIP2}$, which are general inhibitors of the CDKs that regulate the G_1 to S transition. The second class of CKIs is the INK4 family consisting of $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$, which are specific inhibitors of the cyclin D1/CDK4 or CDK6 complexes (Sherr and Roberts, 1999). A third family that regulates the transition from G_1 to S is that of p53, including p53, p63 and p73 (Vousden, 2000). In its

activated, hypophosphorylated form, pRB interacts with E2F thereby repressing transcriptional activity of E2F and hence inhibiting transcription of essential genes for the S phase (Coqueret, 2002; Trimarchi and Lees, 2002; Murray, 2004). Phosphorylation of pRB is in turn controlled by the activity of CDKs and CKIs. It is also known that the AhR interacts with the hypophosphorylated form of pRB (Ge and Elferink, 1998; Elferink et al., 2001). The AhR/pRB complex appears to prevent the normal progression of the G_1 to S phase by blocking E2F-mediated transcription of S phase genes (Puga et al., 2000; Marlowe et al., 2004). Besides its interaction with pRB, the AhR also modulates $p27^{KIP1}$ gene expression and thereby induces a cell cycle arrest in different cell types including developing thymus cells (Kolluri et al., 1999), rat hepatoma cells (Kolluri et al., 1999; Levine-Fridman et al., 2004), human neuronal cells (Jin et al., 2004), and human endothelial cells (Pang et al., 2008). Generally, the induction of $p27^{KIP1}$ is known to negatively regulate G_1 phase progression (Sherr and Roberts, 1995; Sherr and Roberts, 1999). Induction of $p27^{KIP1}$ expression through the AhR results in the prevention of pRB phosphorylation and hence keeps E2F repressed (Huang and Elferink, 2005). In AhR negative fibroblasts, growth promoting genes such as cyclin and CDK genes were significantly downregulated, whereas growth arresting genes like CKIs were upregulated (Chang et al., 2007). Within this thesis these findings were supported in keratinocytes by comparing AhR knockdown HaCaT cells (siAhR HaCaT) to wildtype HaCaT cells. In siAhR HaCaT cells, the protein level of the CKI $p27^{KIP1}$ was increased, whereas the protein level of pRB was decreased compared to wildtype HaCaT cells. The decreased protein level of pRB may result from the fact that transcription of pRB is controlled by E2F family members (Trimarchi and Lees, 2002). Repression of E2F thus results in a reduced expression of pRB. CDK2 activity is required for the cells to enter the S phase and is regulated through $p27^{KIP1}$. Increased protein levels of $p27^{KIP1}$ generally result in the inhibition of CDK2 (Puga et al., 2002). In consistence with the literature a decreased protein level of CDK2 was detected in siAhR HaCaT cells. Protein levels of CDK4 and CDK6 remained unchanged compared to wildtype HaCaT cells. Another difference between wildtype and siAhR HaCaT cells was the protein level of ARNT, which was reduced in siAhR HaCaT cells. Whether this reduction is an artefact due to siRNA transfection or whether there is a coregulation of *AhR* and *ARNT* or *ARNT* is regulated in an AhR dependent manner remains to be identified.

In the presence of exogenous AhR ligands, modulation of cell cycle regulatory proteins was also observed. In skin, results are so far very limited. In human primary keratinocytes, TCDD promotes cell cycle progression in an AhR dependent manner by decreasing the expression levels of the cell cycle regulatory proteins p53 and $p16^{INK4a}$, whereas in normal human dermal fibroblasts TCDD does not enhance proliferation. In theses cells TCDD rather accumulates the cells in the G_0/G_1 phase of the cell cylce through

decreased expression of the inhibitor of DNA binding (ID) proteins ID-1 and ID-3, being involved in regulation of cell proliferation (Akintobi et al., 2007). Moreover, TCDD is able to modulate pRB phosphorylation (Barnes-Ellerbe et al., 2004) and as described above, the AhR interacts with the hypophosphorylated form of pRB. The AhR dependent G_1 arrest of SK-N-SH human neuronal cells induced by TCDD was furthermore accompanied by an increased expression of $p27^{KIP1}$ and the hypophosphorylation of pRB (Jin et al., 2004). TCDD moreover inhibited proliferation of MCF-7 human breast cancer cells, concomitantly with decreases in pRB phosphorylation, cyclin D1 protein levels, and CDK-dependent kinase activities (Wang et al., 1998). In line with these results an increased protein level of $p27^{KIP1}$ was detected after incubation of wildtype HaCaT cells with either eugenol or isoeugenol. Protein levels of pRB as well as of CDK4 and CDK6 were decreased. Interestingly, the protein level of CDK2 remained unaltered 24 h after incubation of HaCaT cells with either eugenol or isoeugenol, although CDK2 activity is directly regulated by $p27^{KIP1}$. It cannot be excluded that changes in the CDK2 protein level may be detectable to a different point of time.

In conclusion, siAhR HaCaT cells exhibit nearly the same molecular characteristics concerning proliferation than wildtype HaCaT cells after treatment with either eugenol or isoeugenol.

5.2.5 Possible role of feedback mechanisms by AhR target genes

As mentioned in section 5.1, both eugenol and isoeugenol induced *CYP1A1* and *AhRR* mRNA in HaCaT cells. Induction of *CYP1A1* through ligand-dependent AhR activation does not necessarily include metabolism of the ligand through CYP1A1. PAHs like B[a]P are metabolized through the further induced CYPs (Nguyen and Bradfield, 2008), whereas TCDD which belongs to the HAHs and is also a strong inducers of CYPs, is hardly metabolized. It is not clear so far whether eugenol and isoeugenol are substrates for CYP1A1. Although the molecular structure of eugenol and isoeugenol is relatively simple and the number of possible metabolites generated through phase I reactions is quite high (Cruciani et al., 2005), metabolism of both substances through CYP1A1 is not shown until now. It remains furthermore unclear whether the induction of *CYP1A1* has an influence on the eugenol/isoeugenol-induced AhR dependent cell cycle arrest in HaCaT cells. Studies have shown that target genes of the AhR like *CYP1A1*, among others are regulated in a cell cycle-dependent manner. The strongest induction of *CYP1A1* by TCDD occurs during late G_1 to early S phase (Santini et al., 2001) and this is one reason for measuring eugenol/isoeugenol-induced *CYP1A1* expression and modulation of cell cycle regulatory proteins in synchronized HaCaT cells. Serum-mediated release of G_0/G_1-synchronized 5L rat hepatoma cells into the cell cycle results in transient activation of the AhR and subsequent

CYP1A1 expression, followed by progression of the cells into the S phase (Levine-Fridman et al., 2004). In contrast, treatment of the same cells with TCDD results in sustained AhR activation, increased $p27^{KIP1}$ expression, and G_1 arrest. Simultaneous treatment of G_0/G_1-synchronized 5L cells with serum and the CYP1A1 inhibitor 1-(1-propynyl)pyrene (1-PP) triggers sustained AhR activation and $p27^{KIP1}$ induction, similar to the action of TCDD alone. Thus, in response to serum stimulation the activity of CYP1A1 appears to negatively regulate the duration of AhR activation through metabolizing a yet unknown endogenous AhR ligand and CYP1A1 substrate, allowing cells to progress through the cell cycle. Possibly, the lack of metabolism of TCDD and other persistent AhR ligands is responsible not only for the sustained induction of AhR activation but also for the induction of cell cycle arrest. Whether eugenol and isoeugenol are also persistent AhR ligands, assuming they do directly interact with the receptor, remains to be identified. Prolonged cell cycle arrest indeed might be a hint for not being metabolized.

The *AhRR* is inducible in an AhR dependent manner (Mimura et al., 1999) (cf. section 1.2.2) and besides *CYP1A1* induction, eugenol and isoeugenol also induced *AhRR* mRNA levels in HaCaT cells. The AhRR functions on the one hand, as a negative regulator of AhR by competing with AhR for ARNT binding and moreover represses XREs in a complex with ARNT. On the other hand, current studies point to a role of the AhRR in chemoprevention. Overexpression of AhRR in the breast cancer cell line MCF-7 results in growth inhibition (Kanno et al., 2006) and evidence from *in vitro* and *in vivo* experiments support a critical role of *AhRR* as a putative tumour suppressor gene in several types of human cancer (Zudaire et al., 2008). Whether the AhRR also impacts on the eugenol/isoeugenol-induced cell cycle arrest and whether the AhRR is a potential mediator of the known putative chemopreventive properties of eugenol and isoeugenol deserves further investigations.

5.2.6 Hypothetical model for the physiological function of the AhR in HaCaT cells

Due to the fact that the AhR promotes cell cycle progression in HaCaT cells in the absence of exogenous ligands, one can speculate that this is the ultimate physiological function of the receptor in this cell line. A hypothetical model describing this function might be the following: In consistence with the known facts from literature mentioned above (cf. section 5.2.4), a putative endogenous ligand may bind to the AhR in the G_1 phase of the cell cycle, thereby transiently activating the receptor and provoking its translocation into the nucleus. Once there, the receptor may function as transcription factor (cf. section 1.2.2), inducing/upregulating target gene expression, among them *CYP1A1*. Assuming that the AhR has the highest affinity to promoters of target genes like *CYP1A1* it might be possible that those promoters to which the AhR has a lower affinity may not be transactivated due to its transient activation. Consequently only target genes like *CYP1A1* might be induced. The putative physiological role of CYP1A1 could be the metabolism of the endogenous ligand, thereby deactivating the AhR. A further putative physiological function may be the general metabolism of ubiquitous biomolecules potentially disturbing the process of DNA synthesis and following mitosis. Due to this putative mechanism, progression of cell cycle would be promoted. Situation changes in the presence of exogenous ligands like eugenol and isoeugenol, assuming they are indeed ligands of the AhR. Eugenol and isoeugenol may have a higher affinity to the AhR thereby displacing the putative endogenous ligand. Binding of eugenol/isoeugenol may result in sustained AhR activation, followed by sustained transactivation of target genes. Prolonged activation of the AhR may furthermore result in transactivation of promoters to which the AhR has a low affinity. Consequently, not only *CYP1A1* is induced but also genes like $p27^{KIP1}$ for instance, being definitely the case in HaCaT cells. Eugenol and isoeugenol are not necessarily substrates for CYP1A1 and may therefore induce prolonged activation of the AhR. As a consequence the AhR might be distracted from its ultimate function in promoting cell cycle progression in the presence of these substances, resulting in the induction of a cell cycle arrest. This interruption of cell proliferation might be a protective mechanism whereby the cell responds to exogenous effects.

5.3 Overall conclusion

To conclude, eugenol and isoeugenol induced the translocation of the AhR from the cytoplasm into the nucleus of HaCaT cells and thereby increased the expression of the AhR target genes *CYP1A1* and *AhRR*. Due to the eugenol/isoeugenol-induced translocation of the AhR into the nucleus, the receptor seemed to be distracted from its physiological function in promoting cell cycle progression of HaCaT cells, as demonstrated by AhR knockdown keratinocytes. In the absence of exogenous ligands the AhR promotes cell cycle progression in many tissues and this knowledge was expanded on skin-derived cells within this thesis. In contrast, in some tissues AhR deficiency is accompanied with accelerated proliferation. Moreover, activation of the AhR by exogenous ligands, as well as by eugenol and isoeugenol results in perturbation of the cell cycle at most in terms of a G_0/G_1 arrest, diminished capacity for DNA replication, and inhibition of cell proliferation. Hence, the AhR has diametrically opposed pro-proliferative and anti-proliferative functions which have not been reconciled at the molecular level until now.

6. Summary

As an interface between an individual and its environment, the skin is a major site of direct exposure to exogenous substances. Once absorbed, these substances may interact with different biomolecules within the skin. The aryl hydrocarbon receptor (AhR) signalling pathway is one mechanism whereby the skin responds to exposures, predominantly through the induction or upregulation of metabolizing enzymes. One known physiological role of the AhR in many tissues is its involvement in the control of cell cycle progression. In contrast to xenobiotic metabolism, almost nothing is known about this function in skin. Moreover, the question whether frequently used naturally occurring phenolic derivatives like eugenol and isoeugenol impact on the AhR within the skin has rarely been studied so far. Eugenol and isoeugenol are due to their odour referred to as fragrances. The ubiquitous distribution of eugenol and isoeugenol results in an almost unavoidable contact with these substances in our daily lives. Despite this fact, their molecular mechanisms of action in skin are poorly understood. There is evidence supporting the hypothesis that these substances may impact on the AhR. On the one hand, eugenol is shown to induce *CYP1A1*, a well-known target gene of the AhR and on the other hand, their known anti-proliferative properties might also be mediated by the AhR, based on its physiological function. In order to proof this hypothesis, it was investigated whether eugenol and isoeugenol impact on the AhR signalling pathway in skin cells. The nuclear translocation of the AhR is a well-known consequence of AhR/ligand interactions. Therefore, it was studied whether both substances cause a translocation of the AhR from the cytoplasm of the cell into the nucleus. Additionally, the impact of eugenol and isoeugenol on AhR target gene expression was examined and moreover the effect of both substances on cell cycle progression. In order to reveal the physiological role of the AhR in skin cells, cell cycle progression of wildtype skin cells was compared to that of AhR knockdown skin cells.

Results revealed that eugenol as well as isoeugenol interacts with the AhR in skin cells. Both substances caused the translocation of the AhR into the nucleus and induced the expression of the well-known AhR target genes *CYP1A1* and *AhRR*. Furthermore, eugenol and isoeugenol exhibited impact on cell cycle progression. Both substances altered cell cycle state distribution due to a prolongation of the G_0/G_1 phase of the cell cycle, inhibited DNA synthesis and thereby reduced cell numbers. Additionally, it was possible to detect that these antiproliferative properties are mediated through the AhR. Moreover, protein levels of cell cycle regulatory polypeptides involved in the transition from the G_0/G_1 to the S phase of the cell cycle were analyzed. Eugenol as well as isoeugenol increased the protein level of the cyclin dependent kinase inhibitor (CKI) $p27^{KIP1}$, whereas protein levels of pRB and the cyclin dependent kinases (CDK) CDK4 and CDK6 were decreased. Protein levels of CDK2 and

AhR nuclear translocator (ARNT) remained unchanged. The comparison of wildtype cells to AhR knockdown cells revealed an impact of the AhR on cell cycle progression in skin cells in the absence of exogenous ligands. AhR knockdown cells exhibited a slower progression through the cell cycle caused by an altered cell cycle state distribution when compared to wildtype cells. These effects were accompanied by an accumulation of cells in the G_0/G_1 phase of the cell cycle and a decreased DNA synthesis rate. Modulation of cell cycle regulatory proteins involved in the transition from the G_0/G_1 to the S phase of the cell cycle was altered in AhR knockdown cells as well. The protein level of $p27^{KIP1}$ was increased, whereas protein levels of pRB and CDK2 were decreased and protein levels of CDK4 and CDK6 remained unchanged. In addition, the protein level of ARNT was decreased.

To conclude, eugenol as well as isoeugenol was able to impact on the AhR signalling pathway in skin cells. Their molecular mechanisms of action are similar to those of classical AhR ligands, although their structural characteristics strongly differ from that of these ligands. In the absence of exogenous ligands the AhR promotes cell cycle progression in many tissues and this knowledge could be expanded on skin-derived cells within the scope of this thesis.

7. References

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Declaration

Hereby, I declare that the presented thesis *The impact of the phenolic derivatives eugenol and isoeugenol on the Aryl Hydrocarbon Receptor signalling pathway in human keratinocytes (HaCaT)* is my own work and that it has not been submitted to any other university for any degree or examination.

Hiermit erkläre ich, dass die vorliegende Arbeit *The impact of the phenolic derivatives eugenol and isoeugenol on the Aryl Hydrocarbon Receptor signalling pathway in human keratinocytes (HaCaT)* selbständig von mir verfasst wurde und bisher weder im ganzen noch in Teilen in dieser Fakultät oder einer anderen akademische Institution eingereicht worden ist.

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Curriculum Vitae

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Publications

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Summary in German

Die Haut stellt eine Grenzfläche zwischen Individuen und deren Umwelt dar und ist als solche täglich einer Vielzahl von Chemikalien ausgesetzt. Diese Chemikalien können von der Haut absorbiert werden und je nach Substanz mit verschiedenen, in der Haut vorhandenen Molekülen reagieren. Der Arylhydrokarbon Rezeptor (AhR) Signalweg ist ein möglicher Mechanismus, mit dem die Haut auf solche Substanzen reagiert. Die am besten untersuchte Funktion des AhR ist die Kontrolle der Expression von Fremdstoff-metabolisierenden Enzymen. Die physiologische Funktion des AhR ist jedoch weitestgehend unbekannt. In einigen Geweben zumindest nimmt dieser Rezeptor Einfluss auf die Kontrolle des Zellzyklus`. Über die physiologische Funktion des AhR in der Haut ist wenig bekannt. Darüber hinaus wurde die Frage, ob natürlich vorkommende Substanzen, wie beispielsweise die Phenolderivate Eugenol und Isoeugenol, Einfluss auf diesen Signalweg in der Haut nehmen können bisher nur unzureichend untersucht. Eugenol und Isoeugenol werden aufgrund ihres aromatischen Geruches als Duftstoffe bezeichnet. Duftstoffe sind in vielen kosmetischen und pharmazeutischen Produkten enthalten, sind Bestandteile von Reinigungs- und Haushaltsmitteln und finden Anwendung als Geschmacksverstärker in Lebensmitteln. Des Weiteren sind sie häufig in Lacken und Farben enthalten. Obwohl sich der Kontakt mit Eugenol und Isoeugenol aufgrund ihrer ubiquitären Verbreitung im täglichen Leben kaum vermeiden lässt, sind deren molekularen Wirkmechanismen innerhalb der Haut nicht geklärt. Es gibt Hinweise darauf, dass diese beiden Moleküle den AhR Signalweg beeinflussen. Zum einen konnte bereits gezeigt werden, dass Eugenol die Expression von Cytochrom P450 1A1 (*CYP1A1*) induziert, ein bekanntes Zielgen des AhR. Des Weiteren ist es aufgrund der oben genannten physiologischen Funktion des AhR denkbar, dass die bekannten antiproliferativen Eigenschaften beider Moleküle über diesen Rezeptor vermittelt werden. Aus diesem Grund wurde die Wirkung von Eugenol und Isoeugenol auf den AhR Signalweg in Hautzellen studiert. Zudem sollte der Einfluss des AhR auf die Proliferation von Hautzellen mit Hilfe einer AhR-defizienten Zelllinie untersucht werden. Im Rahmen dieser Arbeit konnte zum ersten Mal ein Einfluss von Eugenol und Isoeugenol auf den AhR Signalweg in Hautzellen gezeigt werden. Beide Moleküle bewirkten die Translokation des Rezeptors vom Cytoplasma der Zellen in den Zellkern und die Induktion der AhR Zielgene *CYP1A1* und AhR Repressor (*AhRR*). Zudem verursachten beide Moleküle einen AhR-abhängigen Zellzyklusarrest, modulierten die Expression von Zellzyklus-regulatorischen Proteinen und hemmten folglich die Proliferation der untersuchten Zellen. Des Weiteren konnte in den untersuchten Hautzellen gezeigt werden, dass der AhR in Abwesenheit von exogenen Liganden das Fortschreiten des Zellzyklus unterstützt, was seine physiologische Beteiligung an der Zellzykluskontrolle bestätigt.