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Natural furocoumarins as inducers and inhibitors of cytochrome P450 1A1 in rat hepatocytes

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Abstract

Furocoumarins are natural plant constituents present in medicinal plants and in a variety of foods such as grapefruit juice. They are phototoxic and act as potent inhibitors of drug metabolism. We have investigated the interaction of four furocoumarins angelicin, bergamottin, isopimpinellin, and 8-methoxypsoralen with the expression and activity of aryl hydrocarbon receptor (AhR)-regulated CYP1A1 in rat hepatocytes in primary culture, both in the presence and absence of light. In intact hepatocytes pretreated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and in microsomes isolated thereof, all furocoumarins tested acted as potent inhibitors of CYP1A1 activity bergamottin being the most potent inhibitor in microsomes with an IC_{50} of 10 nM in the presence and 60 nM in the absence of light. 8-Methoxypsoralen and angelicin led to a significant induction of CYP1A1 mRNA in hepatocytes, while all furocoumarins except bergamottin increased xenobiotic-responsive element-driven reporter gene expression in transfected H4IIE rat hepatoma cells when light was excluded. Furthermore, all furocoumarins tested induced the expression of endogenous, immunoreactive CYP1A1 protein, primarily in the dark. In conclusion, our results demonstrate that individual furocoumarins present in food and medicinal plants can interfere with AhR-regulated CYP1A1 expression and activity in at least three major ways, i.e., (i) act as highly potent inhibitors of the catalytic activity of CYP1A1 both in the presence and absence of light, (ii) induce CYP1A1 gene expression in the absence of light via activation of the AhR, and (iii) induce CYP1A1 gene expression without activation of the AhR.

Keywords: CYP1A1; Furocoumarins; Induction; Inhibition; Rat hepatocytes; TCDD

1. Introduction

Furocoumarins are biologically active natural compounds found in many plants in particular members of the *Umbelliferae* and *Rutaceae* [1,2]. In food, furocoumarins are present, e.g., in celery (*Apium graveolens L.*), parsnip (*Pastinaca sativa*), parsley (*Petroselium crispum*), carrot (*Daucus carota L.*), orange (*Citrus aurantium* L.), lemon (*Citrus limon*) or limette (*Citrus aurantiifolia*). They are typical phototoxic compounds leading to photodermatitis in combination with UV light exposure [3]. Furthermore, furocoumarins are cytotoxic and mutagenic in mammalian cells in culture [3]. In humans and experimental animals, chronic furocoumarin treatment, in combination with UV light, as used in the therapy of psoriasis, can result in different types of dermal tumors [4].

On the molecular level furocoumarins bind to cellular constituents such as proteins, lipids, etc., can damage lysosomes, lead to the formation of reactive oxygen species, and can contribute to the formation of novel antigens by covalent modification of proteins [5].

Furthermore, furocoumarins are well known for their interference with drug metabolism, in particular with cytochromes P450 (CYP). Tassaneeyakul et al. [6] found that the monomeric 6′,7′-dihydroxybergamottin and related dimers present, e.g., in grapefruit juice, are highly potent inhibitors of CYP3A4 and other CYP enzymes involved in the metabolism of many drugs [7]. Imperatorin

Abbreviations: AhR, arylhydrocarbon receptor; ARNT, arylhydrocarbon receptor nuclear translocator; CYP, cytochrome P450; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; DTT, dithiothreitol; EROD, 7-ethoxyresorufin *O*-deethylase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LDH, lactate dehydrogenase; 5-MOP, 5-methoxypsoralen; 8-MOP, 8-methoxypsoralen; PAH, polycyclic aromatic hydrocarbons; PMSF, phenylmethylsulfonyl fluoride; PROD, 7-pentoxyresorufin *O*-depentylase; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; XRE, xenobiotic-responsive element

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and isopimpinellin act as inhibitors of 7-pentoxyresorufin *O*-dependentylase (PROD) activity catalysed by CYP2B enzymes, while bergamottin and coriandrin inhibit 7-ethoxyresorufin *O*-deethylase (EROD) activity catalyzed by CYP1A enzymes. In fact, moderate consumption of grapefruit juice can result in an increase in bioavailability, maximum plasma concentration and/or elimination half-life of a variety of drugs [8]. Respective warning hints are included in the patient information of a number of medications. Other types of food may have similar effects as indicated by a report on an extended pharmacological action of aminopyrin or paracetamol in mice treated with celery or parsley juice [9].

According to Apseloff et al. [10] 8-methoxypsoralen (8-MOP) can also act as an inducer of total CYP levels in mouse liver and enhance its own biotransformation in liver microsomes isolated from 8-MOP-treated rats [11]. In other studies induction of CYP1A1 was demonstrated in mouse [12] and rat liver [13] after 8-MOP treatment.

CYP1A1 belongs to the family of aryl hydrocarbon receptor (AhR)-regulated genes. The cytoplasmic AhR is a member of the basic-helix-loop-helix PAS (Per/ARNT/Sim) family of nuclear transcription factors. Upon ligand activation it forms a heterodimer with the ARNT protein, and activates transcription of certain genes including the CYP1A1 gene by binding to a xenobiotic-responsive element (XRE) located in the 5′-flanking region of those genes [14]. The environmental pollutant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), one of the most potent AhR agonists, was used in this study as a full agonist.

We have investigated the relative potencies of four major furocoumarins present in certain types of vegetables, the linear furocoumarins bergamottin, isopimpinellin, and 8-MOP, and the angular furocoumarin angelicin, as inhibitors and inducers of CYP1A1 and/or EROD activity in rat hepatocytes in primary culture. In particular, we have carried out experiments in the presence or absence of light to differentiate between effects of furocoumarins after external activation by light and light-independent effects on drug metabolism. Effects on CYP1A gene expression were analyzed on the levels of mRNA, XRE-driven reporter gene expression, and enzyme protein.

2. Materials and methods

2.1. Materials

All chemicals were of reagent grade and were purchased from commercial suppliers. TCDD was from Promochem (Wesel, Germany), angelicin, bergamottin and isopimpinellin were obtained from Roth (Karlsruhe, Germany), 8-MOP from Sigma (Deisenhofen, Germany). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, and penicillin/streptomycin were from Biochrom (Berlin, Germany). The monoclonal anti-rat CYP1A1 antibody was purchased from Rubitec (Bochum, Germany), the secondary anti-mouse antibody from Pierce (Rockfort, IL, USA). H4IIE hepatoma cells were obtained from the American Type Culture Collection (Rockville, MD, USA).

2.2. Light-dependence of effects

All experiments with furocoumarins were either carried out under 'light' or 'dark' conditions as indicated. 'Dark' conditions were maintained by excluding daylight and artificial light with the exception of a weak source of light of longer wavelengths (red bulb). 'Light' conditions were achieved by using 'normal' laboratory conditions, i.e., the presence of daylight and/or artificial light. Light exposure was characterized by a light intensity of 150–240 lm/m^2; 'darkness' was characterized as a light intensity of $<$0.2 lm/m^2, both measured in the middle of the laboratory at a height of 0.85 m.

2.3. Cell culture and treatment

Primary hepatocytes were isolated from male adult Wistar rats (Charles River, Kisslegg, Germany) weighing 150–200 g by collagenase perfusion. Viabilities of hepatocytes were $>$90% as determined by trypan blue exclusion. Cells were grown at 37 °C and 5% CO_2 in DMEM supplemented with 20% fetal bovine serum, 1% penicillin/streptomycin, and 0.1 μM dexamethasone as previously described [15]. After an initial attachment period of 3 h the medium was replaced by fresh medium and substances for treatment, dissolved in DMSO (0.5% final concentration), were added. Cells were incubated for 48 h. H4IIE cells were seeded and treated under identical conditions at semi-confluence. DMSO treatment served as a negative control, while 1 nM TCDD (dissolved in DMSO) was used for positive controls. For cytotoxicity testing, hepatocytes were treated with test compounds or solvent only. After 48 h, medium samples were taken to determine the activity of lactate dehydrogenase (LDH) leaked through cell membranes. The remaining cell monolayer was homogenized by sonification to determine total LDH activity. LDH activities were measured fluorometrically by a kinetic NADH assay (excitation 360 nm, emission 460 nm) according to Moran and Schnellmann [16] in a Fluoroskan Ascent FL plate reader (Labsystems, Frankfurt, Germany).

2.4. Preparation of cell homogenates and microsomes

After incubation cells were washed with cold saline, harvested by scraping off in cold Tris–buffered sucrose solution (10 mM Tris–HCl, 250 mM sucrose; pH 7.4) and centrifuged. Homogenization was performed with a sonifier (Braun, Reutlingen, Germany) at 50 W on ice for 5 s. Microsomal fractions of TCDD-treated primary rat hepatocytes were obtained by a subsequent centrifugation of the

whole homogenate at 9000 × *g* for 10 min. The supernatants were centrifuged at 100,000 × *g* for 30 min and the microsomal pellets were re-suspended in Tris–buffered sucrose solution or sodium phosphate buffer (pH 8.0). Cells cultured in well plate formats were washed as described above, dried and disintegrated by freezing at −80 °C.

2.5. EROD-assay

EROD activity was determined according to the method of Pohl and Fouts [17] using a LS 50 fluorescence spectrometer (Perkin-Elmer, Wellesley, USA). Analysis of EROD activity and protein content in well plate format was measured as described by Kennedy and Jones [18] using a fluorescence plate reader (Fluoroskan Ascent FL, Labsystems, Frankfurt, Germany) with an excitation wavelength of 544 nm and an emission wavelength of 590 nm for resorufin and 390 nm-excitation to 460 nm-emission for fluorescamin.

2.6. Real time PCR analysis

Total RNA was prepared from rat hepatocytes using the phenol/chloroform extraction method as described [19]. Reverse transcription was performed with the iScript™ cDNA Synthesis Kit (BioRad, Munich, Germany) according to the instructions of the manufacturer. For amplification of the CYP1A1 cDNA (GenBank accession no. NM012540) the following primers were used: forward 5′-CCCACAGCACCATAAGAGATACAAGTC-3′ and reverse 5′-GGCCGGAACTCGTTTGGATC-3′. As control, glyceraldehyde phosphate 3-dehydrogenase (GAPDH; GenBank accession no. AF106860) cDNA was amplified with the following primers: forward 5′-TGCACCACCAACTGCTTAGC-3′ and reverse 5′-GGCATGGACTGTGGTCATGAG-3′. Real time PCR reactions were carried out with IQ™ SYBR Green Supermix (BioRad, Munich, Germany) according to the instructions of the manufacturer. The cycling conditions were: initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 1 min, annealing at 59 °C for 1 min, and extension at 72 °C for 1 min before fluorescence data acquisition. A final extension followed at 95 °C over 1 min. Complementary DNA was quantified by comparison of the number of cycles required for amplification of unknown samples with those of a series of cDNA standard dilutions. CYP1A1 mRNA induction was determined by comparative analysis of the CYP1A1 and GAPDH expression.

2.7. Westerns blot analysis

For the preparation of total cell lysates, cell cultures were washed with ice-cold saline, and the cells were harvested by scraping-off in saline. After centrifugation at 3000 × *g* for 10 min the pellet was re-suspended in homogenization buffer (10 mM Tris, 10 mM NaCl, 1.5 mM $MgCl_2 \cdot 6H_2O$, 0.05% NaN_3, 0.1% DTT and 0.1% PMSF) and homogenized with a sonifier for 10 s. 20 μg protein of each sample were fractionated electrophoretically on a 10% SDS-gel. The proteins were transferred to a polyvinylidene difluoride membrane (Immobilon P, Millipore, Billerica, USA) using the semi-dry blotting method. After blocking over night at 4 °C in 5% milk powder dissolved in Tris–buffered saline and 0.1% Tween 20, the membrane was washed with Tris–buffered saline containing 0.1% Tween 20, probed with monoclonal anti-CYP1A1 antibodies for 90 min at room remperature and subsequently incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h. The resulting complexes were visualized using the enhanced chemiluminescence detection method according to the manufacturer's protocol (Western Lightning Kit, Perkin-Elmer, Munich, Germany) measured with a LumiImager™ (Roche, Mannheim Germany). To estimate the level of CYP1A1 protein a densitometric analysis was performed using the TINA 2.09a quantification software (Raytest, Straubenhardt, Germany).

2.8. Transient transfection and luciferase assay

The pGL3-promotor expression plasmid and the control plasmid pRL-SV40, expressing the *Renilla* luciferase gene, were purchased from Promega (Promega, Heidelberg, Germany). The pGL3-XRE reporter gene construct contained a 485 bp fragment of the rat CYP1A1 gene including two XREs. H4IIE cells were co-transfected with 10 μg of the reporter gene construct and 1 μg of the control plasmid using the calcium phosphate co-precipitation method as described previously [20,21]. After transfection, cells were incubated with furocoumarins for 48 h under light or dark conditions. Subsequently, cells were harvested in Reporter Lysis Buffer (Promega, Heidelberg, Germany) and analyzed using the Dual luciferase system (Promega, Heidelberg, Germany) according to the manufacturer's protocol. Luminometrical measurement was performed with the Lumat LB 9507 (Berthold, Wildberg, Germany). The results were expressed as the ratio between the firefly luciferase activity of the reporter gene plasmid and the *Renilla* luciferase activity of the control plasmid.

2.9. Statistical analysis

Statistical analysis was performed using the GraphPad InStat Version 3.00 for Windows 95/NT (GraphPad Software, San Diego, USA). Results are expressed as means ± standard deviation from at least three independent experiments. Statistical significance of the difference between the control and the experimental group was determined by Dunnett's test, one asterisk indicating significant differences from the control group ($P \leq 0.05$), two asterisks indicating very significant differences from the control group ($P \leq 0.01$).

3. Results

In a stepwise approach we aimed at elucidating the complex interaction of furocoumarins with the AhR/CYP1A1 system. In a first series of experiments we analyzed the effects of furocoumarins on the catalytic EROD activity of CYP1A isozymes in rat hepatocytes. The cells were incubated with furocoumarin concentrations between 1 nM and 1 mM over 48 h. Microscopic inspection of the cultures and analysis of LDH leakage did not reveal any signs of toxicity with the exception of 8-MOP which was cytotoxic at 1 mM in the presence of light (not shown). In untreated cells, an EROD activity of 0.47 ± 0.11 pmol/min mg protein was measured. Interestingly, none of the four furocoumarins tested, angelicin, bergamottin, isopimpinellin, and 8-MOP had any significant inducing effect on EROD activity, while the potent inducer TCDD led to a pronounced, more than 200-fold, induction (data not shown). This result was obtained both under normal ambient light conditions and under exclusion of light. All furocoumarins led to a complete inhibition of EROD activity, independent of the mode of addition of the inhibitor, either over 48 h to intact hepatocytes together with TCDD, or for 10 min prior to addition of NADPH to microsomes prepared from TCDD-treated cells (Fig. 1). 100% EROD activity in TCDD-treated hepatocytes was 275 ± 77 pmol/min mg protein (mean of all experiments), in microsomes isolated from TCDD-treated hepatocytes a basal activity of 2157 ± 648 pmol/min mg protein was determined (mean of all experiments). The means of TCDD-induced EROD activities in the four 'treatment groups' did not differ significantly among the cell preparations or among the microsome preparations, respectively.

The analysis of EROD activities over a wide range of furocoumarin concentrations allowed the calculation of complete inhibition curves including half-maximal inhibitory concentrations (IC_{50}-values) and 95% confidence intervals (Table 1).

In intact hepatocytes, 8-MOP was more potent in the dark than in the presence of light, while the opposite was found for bergamottin. For angelicin and isopimpinellin, no significant differences were found. In microsomes bergamottin was the most potent inhibitor under light conditions with an IC_{50} value of 10 nM in the presence of light. In microsomes, all furocoumarins were more potent inhibitors when light was present, the difference

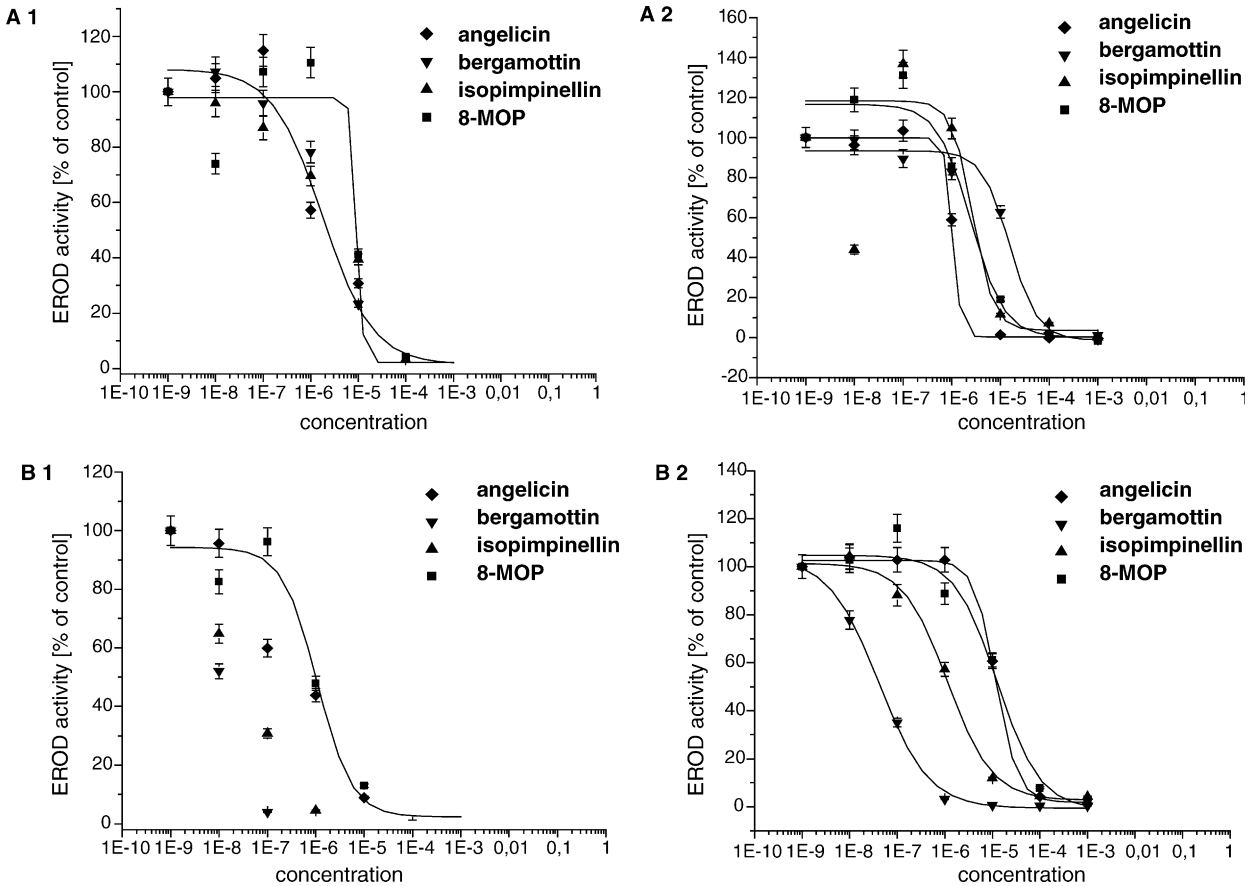

Fig. 1. Effects of angelicin, bergamottin, isopimpinellin, and 8-MOP in co-incubations with 1 nM TCDD on EROD activity in rat hepatocytes in primary culture (A) or in microsomes isolated from TCDD-treated hepatocytes (B). Furocoumarins were added to rat hepatocytes or microsomes under ambient light conditions (A1, B1) or in the dark (A2, B2). Cells were incubated with furocoumarins and/or TCDD over a period of 48 h. Microsomes were pre-incubated with furocoumarins for 10 min before adding NADPH. All furocoumarins and TCDD were dissolved in DMSO. Symbols represent means ± S.D. of at least three independent experiments. Concentration-effect curves were calculated from these data using a log-probit fitting software.

Table 1
IC_{50}-values (μM) of inhibition of EROD activity in TCDD-treated rat heptaocytes in primary culture or in microsomes isolated thereof

Furocoumarin	Heaptocytes		Microsomes	
	Light	Dark	Light	Dark
Angelicin	1.86 ± 1.13	1.36 ± 0.75	0.31 ± 0.22	11.7 ± 0.92[a]
Bergamottin	1.80 ± 0.32	15.66 ± 6.05[a]	0.010 ± 0.0001	0.057 ± 0.0016[a]
Isopimpinellin	5.40 ± 1.92	2.39 ± 4.60	0.015 ± 0.0003	1.36 ± 0.31[a]
8-MOP	9.34 ± 0.06	2.47 ± 1.31[a]	1.07 ± 0.40	2.44 ± 5.79[a]

Furocoumarins were added under ambient light conditions or in the dark as indicated. Cells were incubated with furocoumarins and/or TCDD over a period of 48 h. Microsomes were pre-incubated with furocoumarins for 10 min before adding NADPH. All fourocoumarins and TCDD were dissolved in DMSO. Data represent means ± standard deviations from $n \geq 3$ independent experiments.

[a] significantly different from the corresponding mean in the presence of light ($p < 0.05$).

in potency between light and dark being about six-fold for bergamottin, 12-fold for 8-MOP, 18-fold for isopimpinellin, and 38-fold for angelicin (Table 1).

In a next series of experiments we addressed the question if furocoumarins, which were inactive as inducers of EROD activity, had any effect on the expression of the CYP1A1 gene. Surprisingly, it was found that 8-MOP was as effective as TCDD as inducer of CYP1A1 mRNA (Fig. 2A) when applied at a concentration of 1 mM, in the presence of light being about twice as effective as in the

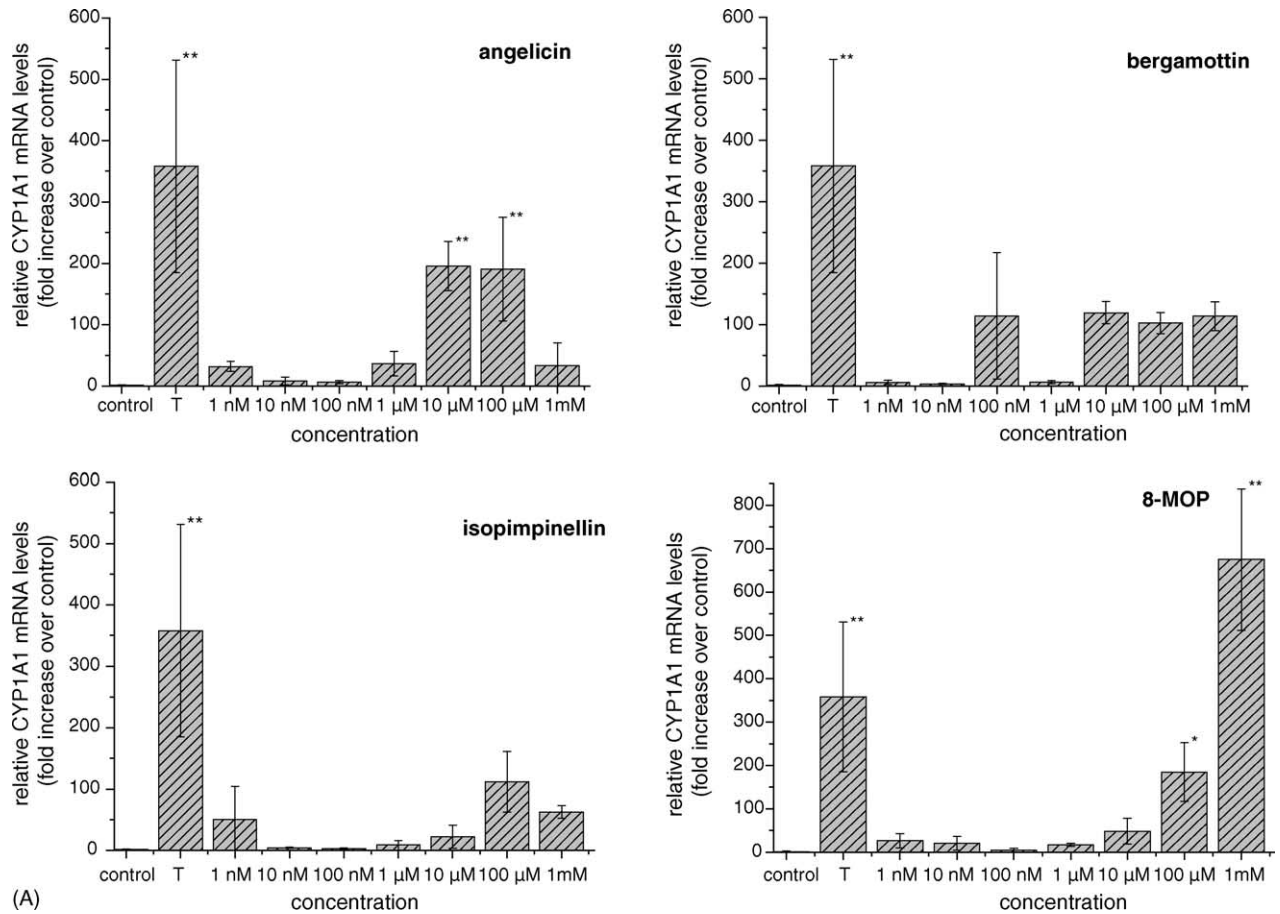

Fig. 2. (A) Effects of angelicin, bergamottin, isopimpinellin, and 8-MOP on CYP1A1 mRNA levels in rat hepatocytes in primary culture under ambient light conditions, in relation to DMSO (vehicle)-treated controls. Cells were incubated with various concentrations of furocoumarins as indicated over a period of 48 h, total RNA was isolated and reverse transcribed, and the cDNA was amplified by real time PCR for quantitative analysis as described in Section 2. Positive controls were treated with 1 nM TCDD (T). All furocoumarins and TCDD were dissolved in DMSO. Bars represent means ± S.D. from $n = 3$ independent experiments; asterisks indicate significant differences ($^{*}P < 0.05$; $^{**}P < 0.01$) from the negative control. (B) Effects of angelicin, bergamottin, isopimpinellin, and 8-MOP on CYP1A1 mRNA levels in rat hepatocytes in primary culture in the dark, in relation to DMSO (vehicle)-treated controls. Cells were incubated with various concentrations of furocoumarins as indicated over a period of 48 h, total RNA was isolated and reverse transcribed, and the cDNA was amplified by real time PCR for quantitative analysis as described in Materials and Methods. Positive controls were treated with 1 nM TCDD (T). All furocoumarins and TCDD were dissolved in DMSO. Bars represent means ± S.D. from $n = 3$ independent experiments; asterisks indicate significant differences ($^{*}P < 0.05$; $^{**}P < 0.01$) from the negative control.

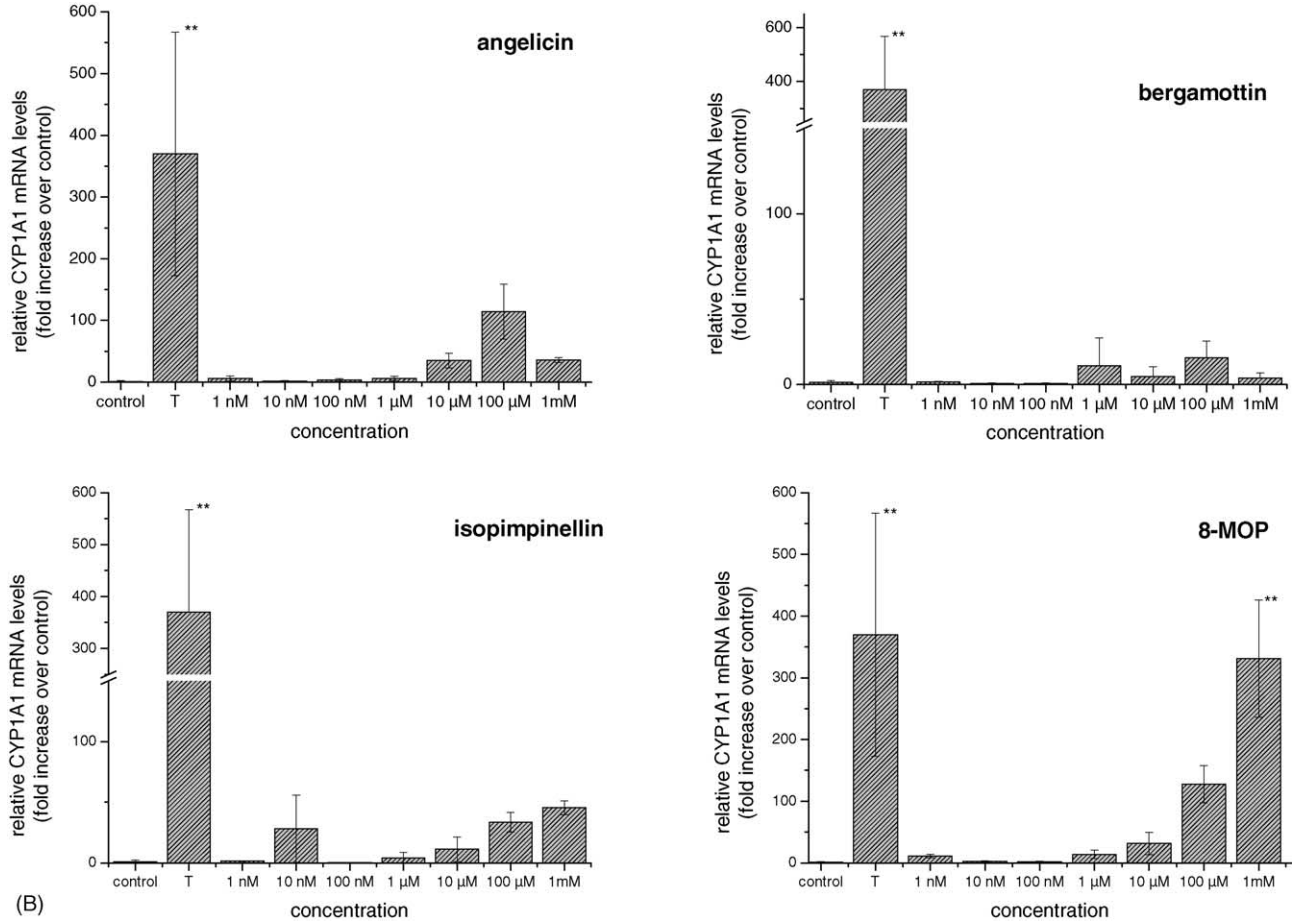

Fig. 2. (*Continued*).

dark (Fig. 2B). The other furocoumarins seemed also to lead to increased CYP1A1 mRNA levels. This effect was statistically significant for angelicin in the presence of light. Induction of other AhR-regulated CYP enzymes cannot be excluded. The primers used, however, identified only one transcript which was assumed to be derived from CYP1A1.

Western blot analysis using monoclonal anti-CYP1A1 antibodies revealed two immunoreactive bands at approximately 55.5 and 53 kDa. Analysis of immunoreactive bands showed that all furocoumarins seemed to lead to a slight increase in enzyme protein(s) when added in the presence of light, the effect being statistically significant for isopimpinellin (Fig. 3). In the dark, however, all furocoumarins were highly effective inducers of CYP1A1 protein(s) with efficacies being in the same range as that of TCDD (Fig. 4). The EC_{50}-values of induction were estimated to be in the range of 1–10 μM.

Finally, we carried out assays to analyze the ability of furocoumarins to activate XRE-driven reporter gene expression in transfected H4IIE rat hepatoma cells. The full agonistic activity of this system was tested with 1 nM TCDD (Fig. 5). In the presence of light, the furocoumarins were completely inactive. In the absence of light, however, all furocoumarins with the exception of bergamottin led to a significant induction of reporter gene expression thus acting as partial AhR agonists.

4. Discussion

In the absence of light, furocoumarins are rapidly distributed throughout the cell [22]. Planar furocoumarins can intercalate between DNA base pairs thus forming a psoralen-DNA-complex. Upon irradiation, extensive binding of furocoumarins to cellular DNA, lipids, and proteins was shown [5,23–25]. Examples for target proteins are bovine serum albumin, glutamate dehydrogenases, lysozyme, nuclear histones, ribonucelases, and *E. coli* DNA polymerases which were shown to bind photo-activated 8-MOP covalently [26].

The interference of furocoumarins with the metabolism of xenobiotics has been investigated in a variety of studies. In humans, 8-MOP underlies a saturable first-pass effect after oral exposure [27]. The metabolism of 8-MOP in rat liver microsomes is inducible by pre-treatment of the animals with phenobarbital, or 3-methylcholanthrene [11]. During metabolism extensive covalent binding to microsomal protein occurs which was suggested to be mediated by reactive metabolites such as an

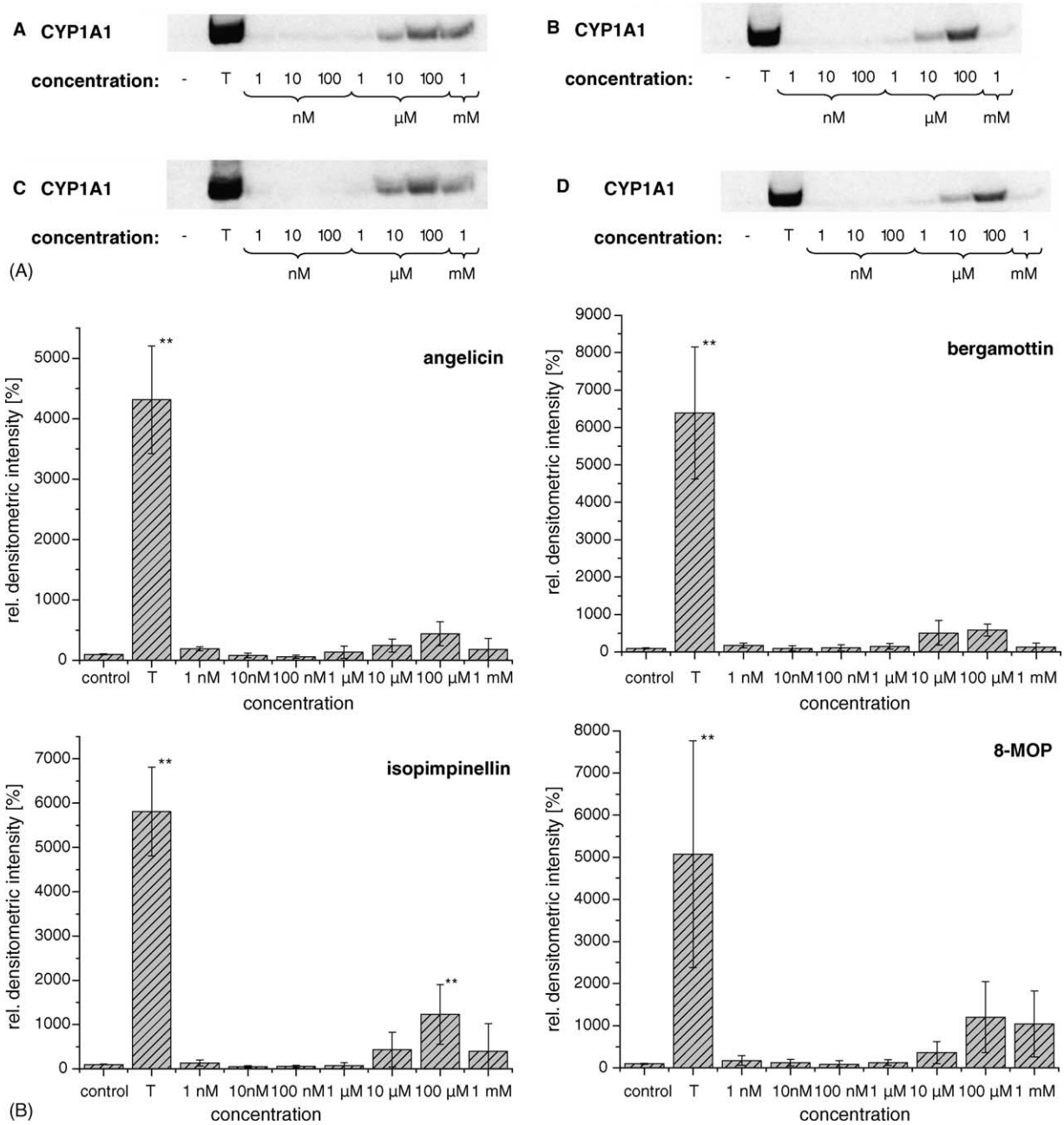

Fig. 3. (A) Representative Western blot analysis of the effects of angelicin (A), bergamottin (B), isopimpinellin (C), and 8-MOP (D) on immunoreactive protein levels (using monoclonal anti-CYP1A1 antibodies) in rat hepatocytes in primary culture under ambient light conditions. Cells were incubated with various concentrations of furocoumarins as indicated over a period of 48 h. Western blot was performed with 20 µg protein sample per lane. Positive controls were treated with 1 nM TCDD (T), negative controls with DMSO (vehicle) only. All furocoumarins and TCDD were dissolved in DMSO. (B) Densitometric analysis of the effects of angelicin, bergamottin, isopimpinellin, and 8-MOP on immunoreactive protein levels (using monoclonal anti-CYP1A1 antibodies) in rat hepatocytes in primary culture under ambient light conditions. Bars represent means and S.D. from $n \geq 3$ independent Western blot analyses like those shown in A. Cells were incubated with various concentrations of furocoumarins as indicated over a period of 48 h. Positive controls were treated with 1 nM TCDD (T), negative controls with DMSO (vehicle) only. All furocoumarins and TCDD were dissolved in DMSO; $^{**}P < 0.01$, i.e., very significantly different from the negative control.

epoxide formed at the furan moiety [28] or an unsaturated dicarbonyl metabolite [29]. The formation of reactive metabolites is also thought to lead to an irreversible inhibition of CYP enzymes [30,31] associated with covalent binding of furocoumarin metabolites to the enzyme [32,33], i.e., suicide inactivation. Thus, the mechanism leading to covalent interaction of furocoumarins with CYP enzymes can be distinguished from photo-activation since it requires an enzyme-catalyzed transformation of the furocoumarin molecule, i.e., can occur in the absence of light but requires cofactors of CYP-catalyzed metabolism. One major target enzyme for inhibition is CYP3A4 [34,35] but a variety of other enzymes including CYP1A1 and 1A2 were also shown to be inhibited by

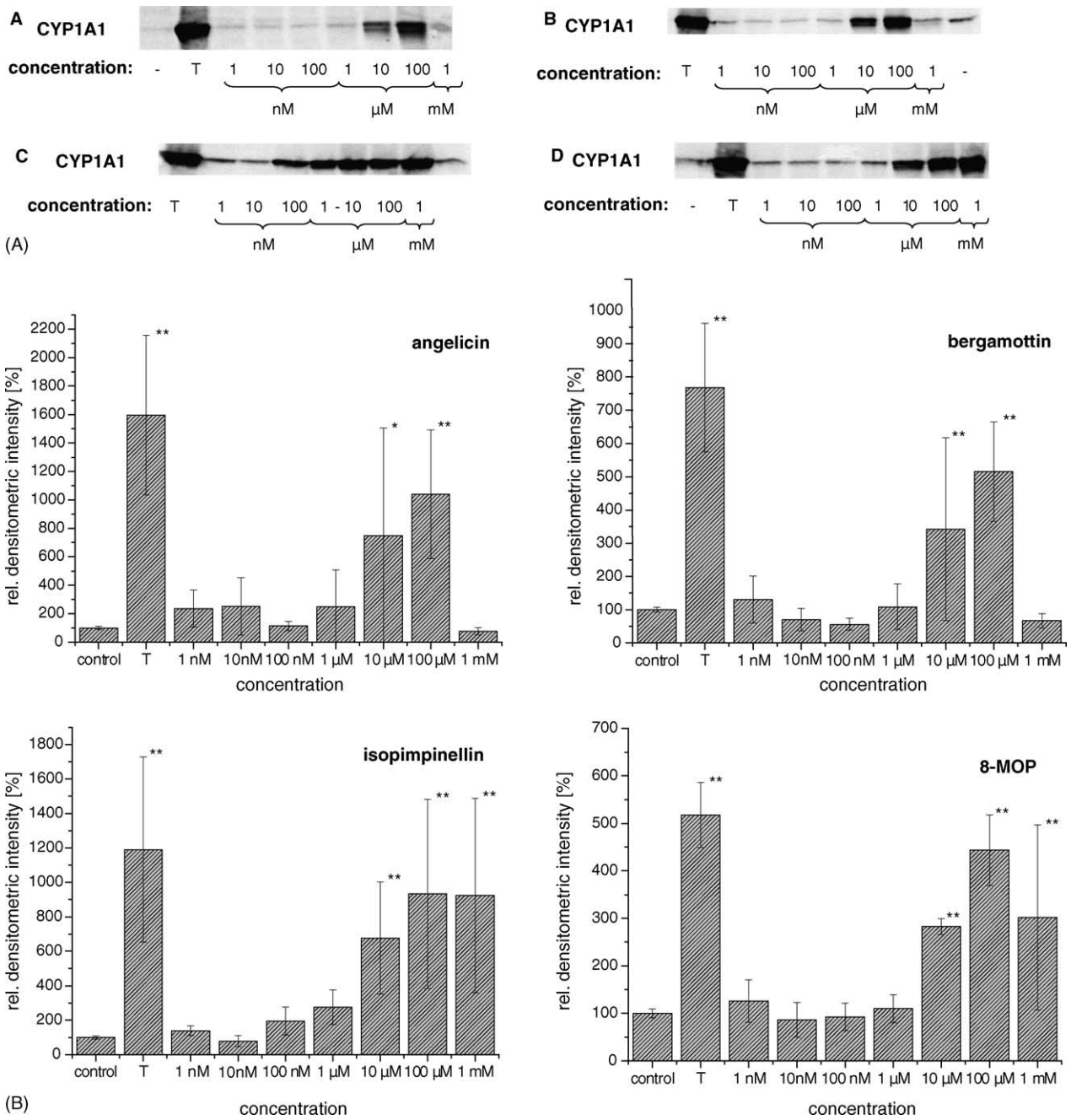

Fig. 4. (A) Representative Western blot analysis of the effects of angelicin (A), bergamottin (B), isopimpinellin (C), and 8-MOP (D) on immunoreactive protein levels (using monoclonal anti-CYP1A1 antibodies) in rat hepatocytes in primary culture in the dark. Cells were incubated with various concentrations of furocoumarins as indicated over a period of 48 h. Western blot was performed with 20 μg protein sample per lane. Positive controls were treated with 1 nM TCDD (T), negative controls with DMSO (vehicle) only. All furocoumarins and TCDD were dissolved in DMSO. (B) Densitometric analysis of the effects of angelicin, bergamottin, isopimpinellin, and 8-MOP on immunoreactive protein levels (using monoclonal anti-CYP1A1 antibodies) in rat hepatocytes in primary culture in the dark. Bars represent means and S.D. from $n \geq 3$ independent Western blot analyses like those shown in A. Cells were incubated with various concentrations of furocoumarins as indicated over a period of 48 h. Positive controls were treated with 1 nM TCDD (T), negative controls with DMSO (vehicle) only. All furocoumarins and TCDD were dissolved in DMSO; $^{**}P < 0.01$, i.e., very significantly different, $^{*}P < 0.05$, i.e., significantly different from the negative control.

furocoumarins [36]. Those authors found that several furocoumarins inhibit EROD activity in mouse liver microsomes. Tassaneeyakul et al. [6] reported that CYP1A2 activity in human liver microsomes was inhibited with bergamottin, 6′,7′-dihydroxybergamottin and two furocoumarin dimers. In human hepatocytes in primary culture bergamottin inhibited β-naphthoflavone-induced EROD activity while the levels of CYP1A1 and 1A2 proteins were actually induced [37]. A weak induction of CYP1A1 was also observed in mouse liver after oral treatment of the animals with 5-MOP alone or in combination with 8-MOP [12].

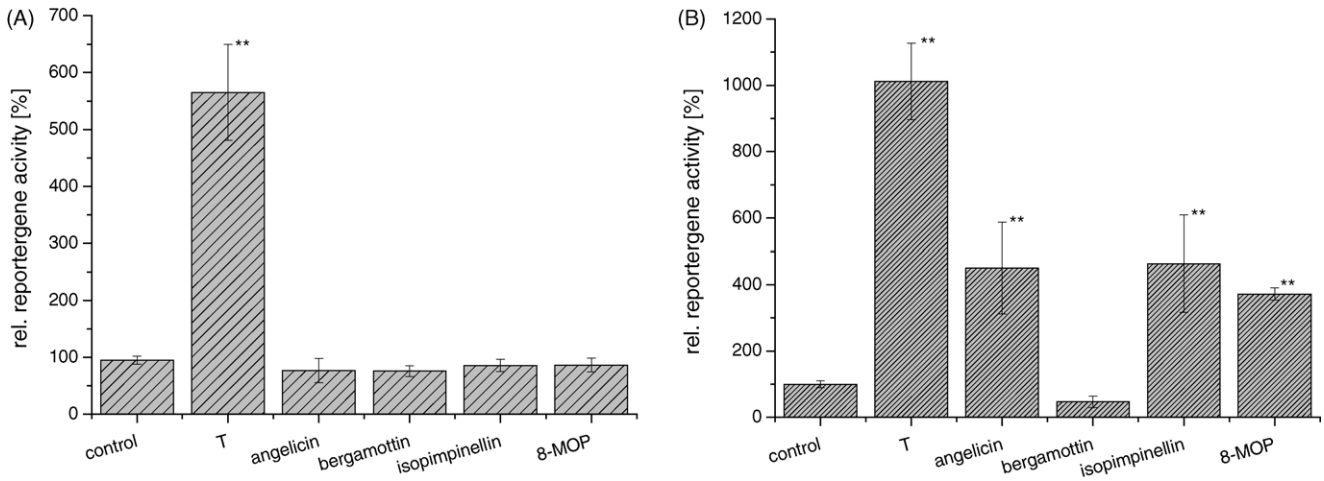

Fig. 5. Relative expression of an XRE-driven reporter gene transfected in H4IIE hepatoma cells. Transfectants were incubated for 48 h in the presence (A) or absence (B) of light with 1 nM TCDD or 100 μM of furocoumarins as indicated. Controls were treated with DMSO (vehicle) only. Bars represent means and S.D. from $n \geq 5$ independent transfection experiments; $^{**}P < 0.01$, i.e., very significantly different from the control.

In this study we focused on the light-dependent interaction of four furocoumarins, angelicin, bergamottin, isopimpinellin, and 8-MOP with AhR-regulated expression of CYP1A1 and EROD activity in rat hepatocytes in primary culture. We could confirm findings in human hepatocytes showing that furocoumarins are potent inhibitors of EROD activity [37].

In the present study the inhibitory effect was analyzed both after addition of furocoumarins to intact rat hepatocytes or to isolated microsomes. In intact hepatocytes lower concentrations of furocoumarins, with the exception of bergamottin, were required for 50% inhibition when light was excluded indicating that the presence of light may reduce the amount of furocoumarin reaching the active site of the enzyme(s). The opposite was found, however, for bergamottin which was a more potent inhibitor in hepatocytes in the presence of light. Interestingly, this was not the case in microsomes. Bergamottin may be trapped in other compartments due to its high lipophilicity thus preventing if from rapidly reaching the active site of the enzyme(s) in intact cells. Under the influence of light, bergamottin may also affect other cellular functions needed for induction of CYP1A1/EROD activity such as DNA binding of the AhR/ARNT complex, transcriptional activation of the CYP1A1 gene, or protein or heme synthesis. In general, the furocoumarins tested were weaker inhibitors in microsomes in the dark than in the presence of light indicating that photoactivation can contribute to inhibition. Bergamottin, however, was a potent EROD inhibitor in microsomes also in the dark with an IC_{50} value of 60 nM which is about 10-fold lower that what was reported for purified human CYP1A1 [33].

Since CYP1A enzymes are involved in the metabolism of a variety of drugs ingestion of furocoumarin-containing food may affect their pharmacokinetics. Furthermore, human and rodent CYP1A1 plays a central role in the metabolic activation of carcinogenic PAH [38]. Previous studies have shown that naturally occurring furocoumarins inhibit the formation of DNA adducts from carcinogenic PAH, e.g., in rodents [39].

In addition to being potent inhibitors of EROD activity and TCDD-mediated EROD induction, we found that furocoumarins can act as inducers of AhR-regulated CYP gene expression. This was not observable, however, on the level of catalytic activity, which is strongly inhibited by furocoumarins. On the level of mRNA, most furocoumarins seemed to lead to a slight increase in CYP1A1 mRNA, while 8-MOP was a highly effective inducer in particular in the absence of light. This finding points to a role of 8-MOP as AhR agonist which was confirmed by expression assays using an XRE-driven reporter gene. This finding is in agreement with previous reports on the induction of 8-MOP metabolism by 8-MOP [11], and the induction of CYP1A1 in mouse [12] and rat liver [13] after 8-MOP treatment. The concentrations required for induction of CYP1A1 mRNA in our experiments were higher by 2–3 orders of magnitude, however, than those leading to suppression of induction of EROD activity. Interestingly, Jeong [13] reported that a maximum increase in hepatic EROD activity was observed in rats 24 h after a single i.p. treatment with 1 mg/kg body weight 8-MOP, after an initial inhibition. This finding suggests that 8-MOP can suppress or enhance EROD activity depending on the dose regimen and half-live of the inducer and the induced enzyme.

Induction was more pronounced on the protein level as revealed by Western blotting, at least when light was excluded. Here, all furocoumarins led to maximum levels of immunoreactive protein(s) at 100 μM, the efficacies being not significantly different. The two bands observed in a variety of blots likely represent CYP1A1 (approximately 55.5 kDa) and CYP1A2 (approximately 53 kDa). Surprisingly, the efficacy was in the same range as that of TCDD.

In an XRE-driven reporter gene assay, all furocoumarins except bergamottin showed inducing activity in the absence of light indicating binding and activation of the AhR. The almost planar, partially aromatic structure of the furocoumarins obviously results in an activation of the AhR pathway while activation by bergamottin may be hampered by the aliphatic side chain of the molecule. Interestingly, all furocoumarins were inactive when the reporter gene assay was carried out in the presence of light suggesting that light-induced covalent modification of the AhR, the AhR/ARNT complex, the AhR/ARNT/DNA complex, and/or the transcriptional machinery can antagonize the induction cascade. In fact, Jeong et al. [40] reported that 8-MOP inhibits the formation of the DNA-protein complex between the AhR and its XRE target as determined by gel mobility shift assays. Alternatively, light may stimulate the breakdown of furocoumarins to products that are less able to activate the AhR signaling pathway.

The findings on potency and efficacy of furocoumarins as inducers of immunoreactive CYP1A1 in rat hepatocytes were in good agreement with the results of the reporter gene assay. In both assays, induction was mainly observed in the absence of light, 8-MOP being the most effective inducer of CYP1A1 mRNA expression. However, bergamottin which was completely inactive as AhR agonist even in the absence of light, was a pronounced inducer of CYP1A1 protein. Bergamottin treatment did neither lead to significantly increased CYP1A1 mRNA levels nor to reporter gene activation, even in the absence of light. Nevertheless, the mechanism of induction by bergamottin may be related to its extraordinary potency as CYP1A1 inhibitor. It can be speculated that such inhibitors can activate a signaling cascade, possible mediated by (an) accumulating endogenous substrate(s) leading to stabilization of the CYP1A1 protein. Alternatively, the inhibitor-bound protein itself may be stabilized towards degradation. The finding that the results of the reporter gene expression do not agree more closely to those of CYP1A1 mRNA expression may be due to the different cell types used as well as to differences in expression of genomic versus plasmid sequences.

In conclusion, our result show that light has a crucial influence on the outcome of experiments on the interaction of furocoumarins with the AhR/CYP1A1 system. In particular, it was found that the four furocoumarins tested are potent inhibitors of the TCDD-mediated induction of EROD activity. The inhibitory potency depends on the experimental model (hepatocytes, microsomes) and the presence of light. The outstanding inhibitory potency of a number of furocoumarins, in particular of bergamottin, is likely to suppress both the metabolism of a number of drugs and the carcinogenicity of PAHs. Further work is needed to elucidate the mechanisms of inhibition of catalytic activity of CYP1A by furocoumarins. All furocoumarins tested except bergamottin are activators of the AhR pathway in the absence of light. Bergamottin was inactive as AhR activator but, nevertheless, also induced CYP1A1 protein indicating an AhR-independent mechanism of induction. Further experiments are needed to clarify if and how the inhibitory and the inductive effects on CYP1A1 are operative in humans and which effect prevails under dietary exposure to natural furocoumarins in food.

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