FUROCOUMARINS AS INHIBITORS AND INDUCERS OF AH-RECEPTOR REGULATED CYP1A ACTIVITY

Annette Baumgart, Melanie Schmidt, Hans-Joachim Schmitz, and Dieter Schrenk

Department of Food Chemistry and Environmental Toxicology, University of Kaiserslautern, 67663 Kaiserslautern, Germany

Introduction

Furocoumarins are present in many fruits and vegetables as natural constituents. Plants with relatively high natural furocoumarin levels are mainly found among rutaceae and umbelliferae[1, 2]. Furocoumarins are well-known to act as phototoxicants in combination with UV irradiation[3, 4, 5] exhibiting cytotoxic and mutagenic properties. Possible mechanisms leading to adverse effects include binding to cellular constituents[6], lysosomal damage[7], generation of reactive oxygen species[8], and formation of novel antigens through covalent modification of DNA[9]. Even in the absence of light, furocoumarins such as 8-MOP have been reported to exert toxic effects on liver, testes and epididymis in rats[10]. Furthermore, a number of furocoumarins act as inhibitors of drug metabolizing enzymes. 6‘,7‘-Dihydroxybergamottin and related furocoumarin dimers found, e.g. in grapefruit juice, act as highly potent inhibitors of cytochrome P450 (CYP) 3A and other CYP isozymes[11] affecting drug metabolism. Little is known on the interaction of furocoumarins with the Aryl hydrocarbon receptor (AhR) and the AhR-regulated CYP1A isozymes.

Methods and Materials

Primary rat hepatocytes were prepared from male Wistar rats weighing 150-220 g. Hepatocytes cultured in DMEM including 20 % fetal bovine serum (FCS) were grown in 60mm-diameter plates. Furocoumarins and TCDD dissolved in dimethyl sulfoxide (DMSO) were added. The cells were incubated at 37 °C for 48 hours. As positive control TCDD in a final concentration of 1nM was used. After incubation the cell homogenates were prepared as described previously[12].

To examine the direct effects of furocoumarins rat hepatocytes were incubated with a constant concentration of TCDD for 48 hours. After preparing rat liver microsomes from this samples EROD assay was carried out by preincubation with furocoumarins for 10 minutes.

The catalytic activity of CYP1A in rat hepatocytes was measured as 7-ethoxyresorufin-O-deethylase (EROD) using a spectrofluorometer (Perkin-Elmer LS-5B).

RNA of rat hepatocytes and H4IIE cells treated with furocoumarins for 48 hours was isolated as described[12]. Semiquantitative RT-PCR was performed with gene specific primers to investigate *CYP1A1* mRNA. The reaction was controlled by simultanous amplification of a glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) fragment.

H4IIE cells and Hepa1 cells were cultured as described above. Cells were incubated for 48 hours with furocoumarin, DMSO and TCDD as positive control. Preparation of nuclear extracts was performed as described previously[13].

Gel mobility shift assays were performed with a double-stranded synthetic oligonucleotide labelled with [g-^{32}P]ATP using T4 polynucleotide kinase (Promega; Heidelberg, Germany). XRE binding reactions were performed in a volume of 20 µl containing 30mM HEPES (pH 7.9), 2.25mM $MgCl_2$, 0.2mM EDTA, 200mM KCl, 20% glycerol, 0.12mM PMSF, 0.5mM dithiothreitol (DTT), 1µg poly(dI-

dC) and 20-25µg of nuclear extract protein. All compounds were incubated at room temperature for 15 min prior to the addition of the double-stranded oligonucleotide. The incubation was continued for a further 15 min. DNA-protein complexes were loaded on a 4 % non-denaturing polyacrylamide gel in 1x TGE at 4 °C and electrophoresed at 170V. Protein-DNA complexes were detected by autoradiography of the dried gel..

H4IIE cells were grown in DMEM supplemented with 20 % FCS and 1 % penicillin / streptomycin. The cells were plated in 60mm-diameter plates. After 24 hours the cells were transiently co-transfected with reporter gene construct containing xenobiotic responsive elements (XREs) of the rat CYP1A1 and the control plasmid pRL-SV40 (Promega, Heidelberg, Germany), expressing the renilla luciferase gene, using the calcium phosphate co-precipitation method as described previously[14]. Reporter gene assays were performed using the Dual luciferase™ system (Promega; Heidelberg, Germany). Cell homogenates were analysed luminometrically (Lumat LB 9507, Berthold; Wildberg, Germany) according to the instructions of the manufacturer. After background correction (activities in untreated cells) relative reporter gene activities were determined by dividing the firefly luciferase activity (reporter gene) by the renilla luciferase activity (control gene).

Results and Discussion

All furocoumarins investigated showed inhibition of CYP1A-mediated EROD activity that was induced by 1 nM TCDD. The IC_{50} values are in the micromolar range.

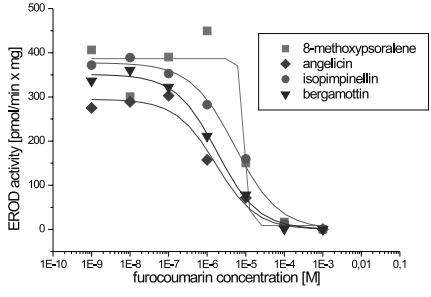

Figure 1. EROD activity of rat hepatocytes coincubated with TCDD [1nM]

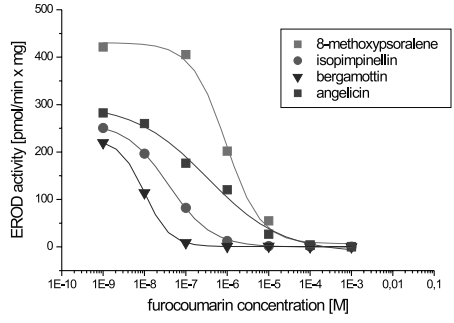

Figure 2. EROD activity of rat liver microsomes pretreated with furocoumarins

Table 1. IC_{50} values of furocoumarins as inhibitors of TCDD-induced EROD activities in rat hepatocytes (with 95 % CI)

Furocoumarin	IC_{50} values [µM]	CI (95 %)
8-MOP	9,34	-
angelicin	1,68	0,65
isopimpinellin	5,19	1,24
bergamottin	1,80	0,32

In microsomes prepared from TCDD-treated primary rat hepatocytes with IC_{50} values in the range from 10 to 90 nM were obtained, giving evidence for a direct inhibition of CYP1A isozymes.

Treatment with the strongly inhibiting furocoumarins angelicin and 8-MOP led to a slight increase in CYP1A1-specific mRNA in rat hepatocytes as could be shown by semiquantitative RT-PCR.

To gain insights into the mechanisms of induction, a reporter gene assay using an XRE-driven luciferase construct (pGL3-XRE), and a gel-electrophoretic mobility shift assay (EMSA) with a ^{32}P-labled XRE-specific oligonucleotide were performed.

The reporter gene assays revealed no significant increase in luciferase activity by treatment of pGL3-XRE-transfected H4IIE rat hepatoma cells with furocoumarins relativ to control (DMSO treatment). Overmore, treatment with angelicin, isopimpinellin, and 5-methoxypsoralene (5-MOP) led to a slight decrease in luciferase activity in comparison to DMSO-treated control.

The mobility shift assay performed with nuclear extracts from H4IIE and Hepa1 cells after treatment with furocoumarins revealed no mobility shift of nuclear proteins by the labled XRE oligonucleotide, whereas TCDD as a positive control clearly showed this effect.

Taken together, these results may suggest that the AhR-ARNT heterodimer not to be involved in the increase of CYP1A1-specific mRNA by the CYP1A-inhibiting furocoumarins in rat primary hepatocytes. AhR-independent increases in expression of CYP1A genes have already been described, and further experiments remain to be performed to elucidate the mechanism of the pathway leading to our observations.

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