Toxicology Letters 193 (2010) 120–123

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

Species-specific activation of nuclear receptors correlates with the response of liver drug metabolizing enzymes to EMD 392949 in vitro

Stefan O. Mueller[a,b,*,1], Yvonne Fery[c,1], Gregor Tuschl[a], Dieter Schrenk[c]

[a] Merck KGaA, Merck Serono, Non-Clinical Development, Toxicology, 64297 Darmstadt, Germany
[b] Institut für Angewandte Biowissenschaften, Karlsruhe Institute of Technology (KIT), Germany
[c] University of Kaiserslautern, Food Chemistry and Toxicology, 67663 Kaiserslautern, Germany

ARTICLE INFO

Article history:
Received 14 September 2009
Received in revised form
15 December 2009
Accepted 17 December 2009
Available online 24 December 2009

Keywords:
Nuclear receptor
Cytochrome P450
PXR
AhR
PPAR

ABSTRACT

We previously reported on the species-specific effects on drug metabolizing enzymes (DME), in particular cytochrome P450-dependent monooxygenases (P450s), by the drug development candidate EMD 392949 (EMD) in vitro and in vivo. Induction of P450s occurs via activation of specific transcription factors such as the arylhydrocarbon receptor (AhR) and the nuclear xenobiotic receptors (NXRs). We analyzed whether the reported species-specific P450 induction by EMD could be related to a specific activation of the CYP1A regulator AhR and the CYP3A regulator pregnane X receptor (PXR) in human and rat cell lines. The human HepG2 and rat H4IIE cell lines exhibited inducibility of CYP1A and 3A and expressed functional AhR as well as PXR. CYP3A was induced by EMD in human HepG2 cells exceeding the level induced by rifampicin, but was not induced in rat H4IIE cells. Regulation of P450s was not related to expression levels of their respective transcription factor, but EMD treatment resulted in a significant reporter gene activation in xenobiotic response enhancer module (XREM)-transfected HepG2 but not H4IIE cells indicating activation of human but not rat PXR. In summary, we showed that the P450 inducing properties of EMD were perfectly reflected by its ability to activate AhR or PXR in a species-specific manner. These findings support the tight correlation of species-specific nuclear receptor activation with P450 induction and foster the use of nuclear receptor activation as a complementary screen to identify cytochrome P450 inducers.

1. Introduction

We previously reported on the species-specific effects of the drug development candidate EMD 392949 (EMD) on drug metabolizing enzymes (DME) in particular cytochrome P450-dependent monooxygenase (P450) in vitro and in vivo (Richert et al., 2008).

Induction of P450s, in most instances, occurs via activation of specific transcription factors such as the arylhydrocarbon receptor (AhR) and the nuclear xenobiotic receptors (NXRs) that regulate gene transcription (Honkakoski and Negishi, 2000). The main transcription factors involved in P450 regulation are: AhR, which mainly regulates CYP1A1/2, constitutive androstane receptor (CAR), which regulates mainly CYP2B/C and CYP3A, and pregnane X receptor (PXR), which regulates predominantly CYP3A (Sueyoshi et al., 1999; Safe, 2001; Sugatani et al., 2005). The regulation of certain clusters of phase II and III enzymes is also mediated via the same NXRs as for the CYPs. Therefore, inducers that cause PXR activation frequently induce CYP3A and certain transporters such as multidrug resistance proteins (MDR/MRPs) in hepatocytes (Richert et al., 2009).

The tight interaction of NXR activation leading to transcription and consequently increased enzyme activity prompted us to analyze whether the reported species-specific P450 induction of EMD could be related to differential activation of the CYP1A regulator AhR and the CYP3A regulator PXR in human vs. rat cell lines.

2. Materials and methods

2.1. Chemicals and reagents

Chemicals used in this study were obtained from Sigma–Aldrich (Schnelldorf, Germany), tetrachlordibenzo-*p*-dioxin (TCDD) was obtained from AccuStandard Inc (New Haven, CT, USA; 99.15% purity). Medium and other reagents for cell culture were from PAA Laboratories GmbH (Coelbe, Germany) unless stated otherwise. Dextran-coated charcoal treated FBS (DCC/FBS) was from Hyclone (Lot AKD11642A,

Abbreviations: AhR, arylhydrocarbon receptor; bp, base pair; DME, drug metabolizing enzyme; DMEM, Dulbecco's modified Eagle's medium; EMD, EMD 392949; FBS, fetal bovine serum; nt, nucleotide(s); NXR, nuclear xenobiotic receptor; P450, cytochrome P450-dependent monooxygenase; PPAR, peroxisome proliferator-activated receptor; PXR, pregnane X receptor; SD, standard deviation; v/v, volume per volume; XRE, xenobiotic response element; XREM, xenobiotic response enhancer module.

* Corresponding author at: Toxicology – Early and Explanatory Toxicology, Merck Serono - Merck KGaA,, Frankfurter Str. 250, 64293 Darmstadt, Germany.
Tel.: +49 6151 72 8517; fax: +49 6151 72 91 8517.
E-mail address: stefan.o.mueller@merck.de (S.O. Mueller).
[1] S.O.M. and Y.F. contributed equally to this work.

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doi:10.1016/j.toxlet.2009.12.014

Table 1
Primers and their annealing temperatures used for SYBR Green real-time PCR.

Gene	GenBank accession #	5′-Sense-3′	3′-Antisense-5′	T_a (°C)
CYP3A1	NM173144	CCAGCAGCACACTTTCCTTTG	GGTGGGAGGTGCCTTATTGG	52
CYP3A4	AF209389	GTCCTACCATAAGGGCTTTTG	AGGCCTCCGGTTTGTGA	50
m/r/h CYP1A1	NM_012540/NM_001136059/NM_000499	CCTCTTTGGAGCTGGGTTTG	CCTGTGGGGGATGGTGAA	58
rPXR	NM_052980	CGATTTGCCCTCACCCTGAAGG	CGTCCGTGCTGCTGAATAACTCC	56
hPXR	AF_061056	CCCAGCCTGCTCATAGGTTC	GGGTGTGCTGAGCATTGATG	56.2
h-β-Actin	NM_001101	TGACCCAGATCATGTTTGAGACC	GGATAGCACAGCCTGGATAGC	50-58
r-β-Actin	BC_063166	CAGGGTGTGATGGTGGGTATGGG	TGGTGACAATGCCGTGTTCAATGG	50-58

m, mouse; r, rat; h, human.
Reaction conditions: 95 °C 3 min; 40× (95 °C 1 min, T_a (shown above) 1 min, 72 °C 1 min); 95 °C 1 min; 55 °C 1 min. A melting curve emerging in a gradient from 55 to 95 °C in increasing steps of 0.5 °C verified the single PCR product.

Table 2
Primers and probes used for TaqMan Real-Time PCR.

Gene	GenBank accession #	TaqMan probe	5′-Sense-3′	3′-Antisense-5′
hAhR rAhR	NM_001621/NM_013149108	CTACTCCACTTCAGCCACCGTCCATCCT	TCCACAGTTGGCTTTGTTTGC	TGTGAAGTCCAGTTTGTGTTTGG

h, human; r, rat.
Reaction conditions: 95 °C 15 min; 45× (95 °C 15 s, T_a 60 °C 1 min); 4 °C ∞.

Perbio Science, Bonn, Germany). Cell culture plastics were purchased from Greiner Bio-One GmbH (Frickenhausen, Germany). EMD 392949 ((*S*)-4-o-tolylsulfanyl-2-(4-trifluormethyl-phenoxy)-butyric acid, batch 00195C0, >95% purity) was from Merck Santé (France).

2.2. Cell culture and treatment

HepG2 and H4IIE cells (purchased from the European Collection of Cell Cultures, United Kingdom) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS and 1% penicillin/ streptomycin and plated with a density of 50,000 cells/cm^2 for HepG2 and 20,000 cells/cm^2 for H4IIE. After 24 h medium was replaced by fresh medium with the test compound EMD and incubated for 48 h. DMSO 0.1% (v/v) served as solvent control, dexamethasone (10 μM), rifampicin (10 μM) and TCDD (0.001 μM) as reference inducers (positive controls).

2.3. Messenger RNA (mRNA) preparation and analysis (real-time PCR)

Total RNA was isolated with TRIR (ABgene, Hamburg, Germany) according to the manufacturer's instructions and dissolved in pure water (LiChrosolv, Merck, Germany). The purity and concentrations of the samples were determined and 500 ng RNA were transcribed into cDNA with iScript cDNA Synthesis Kit (Bio-Rad Laboratories GmbH, München, Germany). Twenty-five nanograms of cDNA for detection of CYP3A1/4, CYP1A1, PXR and β-actin were then assayed using ABsolute® QPCR SYBR Green Flourescein Mix (ABgene, Hamburg, Germany). Primer and conditions are shown in Table 1. Twenty-five nanograms of cDNA for detection of AhR were assayed using qPCRMastermix No ROX and primer and probes (Table 2) from Eurogentec Deutschland GmbH (Cologne, Germany) for TaqMan Real-Time PCR. Expression was calculated using the ΔΔCt method as described (Tuschl and Mueller, 2006).

2.4. Transient transfection and luciferase reporter activity assay

For transient transfection assays, cells were seeded in 24-well plates in DMEM with 10% DCC/FBS for HepG2 and in 10% FBS-containing DMEM for H4IIE cells and maintained overnight. Because H4IIE cells were not viable in DCC/FBS, normal FBS was used. The cultures were washed with PBS, and fresh medium with 5% DCC/FBS for HepG2 and 5% FBS for H4IIE cells was added. Analysis of PXR-dependent activation of CYP3A was performed with a XREM luciferase reporter gene construct. This pGL3-XREM3 vector contained three copies of the XREM/NR binding site derived from the human CYP3A4 promoter/enhancer gene (Fery et al., 2009).

AhR-dependent induction of CYP1A1 was analyzed with a pGL3-XRE reporter gene construct containing a 485 bp fragment of the rat CYP1A1 promoter including two XREs as described (Baumgart et al., 2005). Transient transfection was carried out with FuGENE HD (Roche Diagnostics, Germany) according to the manufacturers' instructions. Fifty nanograms of a renilla luciferase reporter vector was co-transfected as an internal standard. After 6 h of transfection, cells were treated with positive controls, the test compound EMD and the vehicle control (DMSO 0.1% (v/v)) for 48 h. The Dual Luciferase Reporter System (Promega Corporation, Madison, WI, USA) according to the manufacturers' instructions was used. Compound-induced fold-induction was assessed as luminescent readings normalized to the internal transfection and cell number control renilla luciferase compared to the vehicle control.

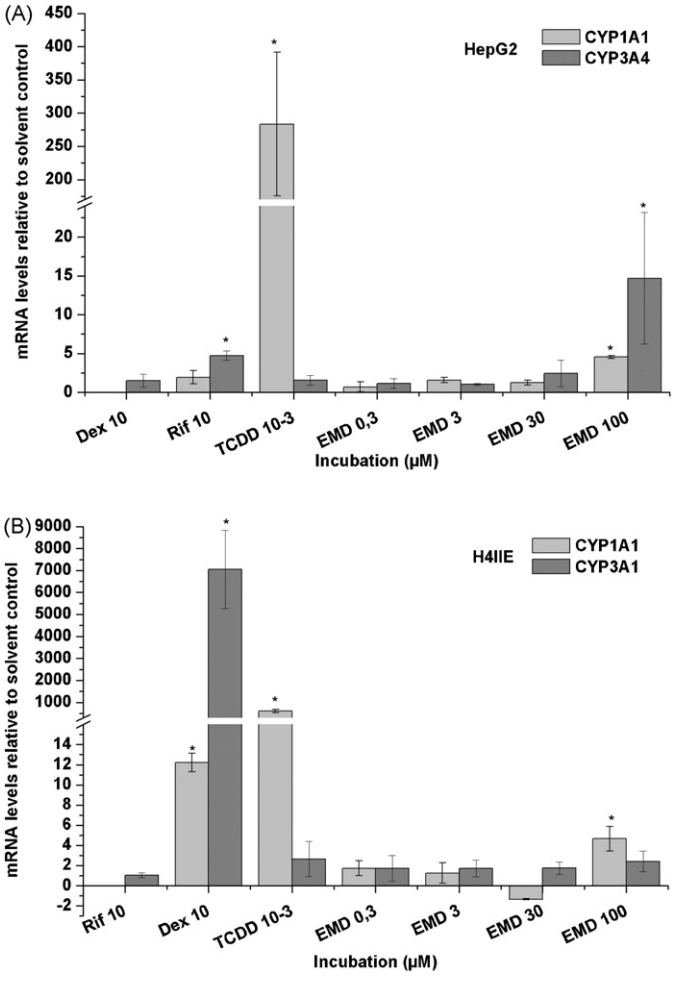

Fig. 1. Effects of EMD on relative CYP1A1 and CYP3A mRNA levels in human HepG2 (A) and in rat H4IIE (B) cells. Prototype inducers dexamethasone, rifampicin and TCDD served as positive controls. Shown are values of fold regulation relative to the untreated control. Bars illustrate mean values from three individual experiments with standard deviation (SD). Please note that the positive *y*-axis shows a break. Asterisk indicates statistically significant difference from the untreated control (*p-values ≤ 0.05).