Propiconazole is an activator of AHR and causes concentration additive effects with an established AHR ligand

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Abstract

Pesticides can exert adverse effects on different target organs via the activation of nuclear receptor pathways. Hepatotoxic effects of the widely used triazole fungicide propiconazole (Pi) are generally attributed to the activation of the ligand-activated transcription factor constitutive androstane receptor (CAR) or the pregnane X receptor (PXR). We now investigated the effects of Pi on the ligand-activated transcription factor aryl hydrocarbon receptor AHR. Therefore, the effects of Pi on AHR-dependent reporter gene transactivation, AHR target gene mRNA and protein expression as well as enzyme activities were examined in HepG2 or HepaRG human hepatoma cells using dual luciferase assays, RT-PCR, mass spectrometry or the ethoxyresorufin-O-deethylase assay, respectively. Additionally mRNA expression and enzyme activity was analyzed in rat liver *ex vivo*. Furthermore, AHR target gene expression was measured in AHR-, CAR-, and PXR- knockout HepaRG cells. Subsequently, we investigated combination effects of Pi and the model AHR ligand benzo[*b*]fluoranthene (BbF) in vitro.

Our results show that Pi activates the AHR in reporter gene assays in human HepG2 cells. This dose-dependent activation of the AHR was subsequently confirmed by real-time RT-PCR analyses of the model AHR target genes *CYP1A1* and *CYP1A2* in human HepaRG cells and in livers of rats treated with Pi for 28 days via the diet. In addition, results of CYP1A1 protein level quantification showed Pi-dependent induction of the enzyme. CYP1A1 enzyme activity measurements demonstrate that higher concentrations of Pi significantly induce CYP1A1-dependent reactions in human HepaRG cells and in rat liver. Gene expression analysis in AHR-knockout HepaRG cells showed no induction of *CYP1A1* and *CYP1A2*, whereas gene expression in CAR-, and PXR-knockout cells was induced. Furthermore, mixture effects of Pi and BbF were observed in human cell lines. Modeling of dose-response curves revealed that these cellular effects can be explained by concentration additivity. In conclusion, our results demonstrate that the triazole Pi is an activator of AHR.

Keywords:

triazole fungicides, nuclear receptor, aryl hydrocarbon receptor, liver toxicity, mixture toxicity, concentration additivity

Abbreviations:

AHR, aryl hydrocarbon receptor; AOP, adverse outcome pathway; BaP, benzo[*a*]pyrene; BbF, benzo[*b*]fluoranthene; CAR, constitutive androstane receptor; CITCO, 6-(4-Chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehydeO-(3,4dichlorobenzyl)oxime; CYP, cytochrome P450; DMSO, Dimethylsulfoxid; EFSA, European Food Safety Authority; EROD, ethoxyresorufin-O-deethylase; FCS, fetal calf serum; HIF-2α, hypoxia-inducible factor 2α; IARC, International Agency for the Research on Cancer; IPA, Ingenuity Pathway Analysis; LBD, ligand binding domain; LLOQ, lower limits of quantification; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PAH, polycyclic aromatic hydrocarbon; Pi, Propiconazole; PPAR, peroxisome proliferator-induced receptor; PXR, pregnane X receptor; RSLC, Rapid Separation LC; SD, standard deviation; TCDD, 2,3,7,8-tetrachlorodibenzo[*p*]dioxin; TCPOBOP, 1,4-Bis-[2-(3,5-dichloropyridyloxy)] benzene; tSIM, targeted Single-Ion-Monitoring

1. Introduction

For assessment of mixture effects, mode of action analysis is critical because the nature of a combination effect (i.e. whether similar or dissimilar action occurs) depends on the molecular interactions of substances with receptors (Kortenkamp et al., 2009). Substances affecting the same target tissue by the same mechanism (e.g. the liver by AHR or PXR activation) are generally assumed to behave in a concentration-additive manner, while diverging target organs and mechanisms would lead to independent action.

Many azole compounds show a hepatotoxic potential in standard toxicity tests. Propiconazole (Pi), a triazole fungicide, for example causes hepatocellular hypertrophy and vacuolization as well as hepatocellular tumors in long-term rodent bioassays (EFSA, 2010). The constitutive androstane receptor (CAR) plays a crucial role in liver toxicity of several azoles, as evidenced by gene expression profiles (Currie et al., 2014; Goetz and Dix, 2009; Heise et al., 2017; Heise et al., 2015) as well as by experiments in knockout mice (Peffer et al., 2007) or humanised mice (Marx-Stoelting et al., 2017). An additionally key mediator of azole-mediated hepatotoxicity is the pregnane X receptor (PXR) (Goetz and Dix, 2009; Hester and Nesnow, 2008). More specifically, it has been suggested that these two receptors are important for the effects also of Pi on the target organ liver (Currie et al., 2014; Hester and Nesnow, 2008). In contrast, the aryl hydrocarbon receptor (AHR) has not yet been described as a key receptor in Pi-mediated liver toxicity in human. Hints on involvement of the AHR in Pi mediated toxicity have been found in other studies involving mice, rats or rainbow trouts (Ghisari et al., 2015; Li et al., 2013; Sun et al., 2005). Other azole fungicides like imazalil, which have been tested for their ability to activate human AHR, were found not to be AHR activators but CYP1A1 inducers (Sergent et al., 2009). However, the imidazole fungicide prochloraz has been proposed to exert its effects by activating AHR (Halwachs et al., 2013; Marx-Stoelting et al., 2017; Rieke et al., 2014) while an involvement of CAR in prochloraz-dependent liver toxicity has also been proposed (Goettel et al., 2015). Since imidazoles and azoles are structurally related and an AHR activation has been shown in other species, the hypothesis that at least some azoles may also interfere with the human

AHR seems plausible and worth of investigation, considering also potential implications for mixture toxicity.

Benzo[*b*]fluoranthene (BbF) is a polycyclic aromatic hydrocarbon (PAH) found as contaminant in food, e.g. in roasted coffee (Jimenez et al., 2014). Like many other PAHs BbF exerts at least parts of its toxicity via the AHR (Andrysik et al., 2007). Interestingly, for some AHR ligands including PAHs like benzo[*a*]pyrene (BaP), species differences have been observed in terms that these substances show a higher affinity to murine AHR (mAHR) as compared to its human counterpart (Flaveny et al., 2009). This emphasizes the need to investigate rodent liver toxicants, known to activate AHR, also for their ability to transactivate the human AHR.

The aim of this work was to analyze AHR activation by Pi in human cell lines and in rats. Furthermore, mixture effects of Pi and the model AHR ligand BbF on this receptor were investigated.

2. Materials and methods

2.1 Test substances

Propiconazole (CAS # 60207-90-1; Batch # CGA64250B; purity 96.10%) was acquired in technical quality from Syngenta AG (Basel, Switzerland) and benzo[*b*]fluoranthene (CAS # 205-99-2; purity 98.00%) from Sigma-Aldrich (Taufkirchen, Germany). For receptor transactivation assays, propiconazole in analytical quality from LGC Standards (Wesel, Germany) (CAS # 60207-90-1; Batch # G144536; purity 99.00%) was used. For dilution of the test substances, DMSO (dimethylsulfoxide) was used, resulting in a final DMSO concentration of 0.2% in the treatment medium. For mixture experiments concentrations of the test substances were halved (10 µM A+B corresponds to 5 µM A + 5 µM B).

2.2 Cultivation of cells

The human hepatocellular carcinoma cells HepaRG were acquired from Biopredic International (Saint Grégoire, France) and cultured as previously described (Gripon et al.,

2002). For a period of two weeks the HepaRG cells were incubated in the proliferation medium which consists of Williams E medium (Pan-Biotech GmbH, Aidenbach, Germany), 10% fetal calf serum (FCS; Pan-Biotech GmbH), 100 U/ml penicillin, 100 µg/mL streptomycin, 0.05% human insulin (PAA Laboratories GmbH, Pasching, Austria) and 50 µM hydrocortisone-hemisuccinate (Sigma-Aldrich). For differentiation of the cells they were incubated for further two weeks in differentiation medium, which encloses the proliferation medium and 1.7% DMSO. Differentiated cells were treated with test substances in phenol red-free Williams E medium (Pan-Biotech GmbH) including the same supplements as the differentiation medium, but only 2% FCS.

The PXR, AHR, and CAR knockout HepaRG cells were purchased from Sigma-Aldrich. The cultivation was done according to the manufacturers' protocol. In brief, cells were thawed in recovery medium (Sigma-Aldrich) and cultivated in recovery medium for two days. Afterwards, cells were cultured for a period of two weeks in maintenance medium (Sigma-Aldrich). It follows one day in pre-induction-medium (Sigma-Aldrich) before the cells were treated in serum-free-induction medium (Sigma-Aldrich).

The incubation of HepG2 cells (ECACC, Porton Down, UK) was performed in DMEM medium (Pan-Biotech GmbH) which included 10% FCS. When the cells had reached a confluency of about 80%, cells were passaged. Treatment with the test substances was performed in phenol red-free DMEM medium (Pan-Biotech GmbH) supplemented with 10% FCS (Pan-Biotech GmbH) for 24 hours. Incubation of both cell lines was done at 37°C and 5% CO_2 in a humidified atmosphere in a Binder cell culture incubator.

2.3 Testing of cell viability

For analysis of cell viability, we used the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay according to standard protocols (Braeuning et al., 2012). The detergent Triton X-100 (0.01%) was used as positive control.

2.4 Animal experiment.

The animal experiment was conducted as previously described (Schmidt et al 2016). In brief, 5-6 week old male Wistar rats were treated with propiconazole via rodent standard diet for 28 days at a dose level of 2.4 ppm, 240 ppm or 2400ppm yielding 181 mg/kg bw/d. Animals were deeply anaesthetized with Sevofluran (Abbot, Germany), finally sacrificed by 95% CO_2 / 5% O_2 and livers were isolated. Directly after isolation, livers were partially frozen on dry ice for subsequent molecular analysis. Additionally, standard pathology and histopathology were performed according to standard principles of the Society for Toxicopathology (STP 2010) as previously reported (Schmidt et al 2016).

2.5 Molecular modelling

The human AHR primary structure was downloaded from the UniProt Protein Knowledgebase database [entry: P35869]. After a protein Blast search of the Protein Data Bank database for a human AHR homolog, the crystal structure of human hypoxia-inducible factor α (HIF-2α) ligand binding domain (LBD) (Erbel et al., 2003) in its apo-form was set as a template. An alignment produced by the Clustal Omega software was manually checked. Comparative model building was carried out by the MOE Homology Model program of the Protein module with default settings. The quality of the final model was carefully checked with the MOE Protein Geometry program. The binding site of the AHR was identified through the MOE Site Finder program, which uses a geometric approach to list putative binding sites in a protein, starting from its three-dimensional structure. Pi and the reference compound 2,3,7,8-tetrachlorodibenzo[*p*]dioxin (TCDD) were downloaded from the PubChem database. Each structure was converted into a three-dimensional structure, and energy minimized, with the MOE Energy Minimize program down to a RMS gradient of 0.05 kcal/mol/$Å^2$. Stereochemistry of each structure was carefully checked. Molecular docking was carried out through the MOE Dock program. The Triangle Matcher placement algorithm was selected and the London dG empirical scoring function was used for sorting the poses. The 30 top-scoring poses were refined through molecular mechanics, considering the AHR as a rigid body, and the refined complexes were scored through the GBVI/WSA dG empirical scoring

function, selecting the five top-scoring poses and estimating their binding free energy (ΔG) to the ligand binding domain of AHR.

2.6 Reporter gene assays

The dual luciferase reporter gene assays were performed using HepG2 cells in 96-well plates. For transiently transfection of the cells, TransIT-LT1 (Mirus Bio LLC, Madison; WI, USA) was used. For each assay, the cells were transfected with two plasmids (see table 1). A detailed description of the generation and the features of the luciferase reporter constructs used in this study is available elsewhere (Schreiber et al., 2006; Schulthess et al., 2015). In brief, a pT81Luc-based firefly luciferase reporter driven by a 1.2 kbp fragment of the wildtype human *CYP1A1* promoter including four functional AHR binding sites was used. In addition, a mutant version of the promoter lacking these AHR-responsive sites (-CDEF) came into operation. Third, a pT81Luc-based artificial promoter construct consisting of three AHR binding sites (3xDRE) was used. For all three assays, cells were co-transfected with a second plasmid, which constitutively expresses Renilla luciferase and served as internal control. After transfection, treatment and lysis of the cells (100 mM potassium phosphate with 0.2% (v/v) Triton X-100, pH 7.8), luminescence was measured using a plate reader (Infinite M200PRO, Tecan, Männedorf, Switzerland) according to the Dual Luciferase Assay protocol provided by the supplier (Promega, Madison, WI, USA).

Table 1: Plasmids used for dual luciferase reporter gene assays in HepG2 cells.

Luciferase assay	**Plasmid**	**Time of incubation**	**Positive control**
hCYP1A1-promoter	pT81Luc-hCYP1A1	48 h	10 µM BbF
	pcDNA3-RLuc		
hCYP1A1 (-CDEF)-promoter	pT81Luc-hCYP1A1-CDEF	48 h	10 µM BbF
	pcDNA3-RLuc		
3xDRE	pT81Luc-3xDRE	24 h	10 µM BbF
	pcDNA3-RLuc		

2.7 Gene expression analysis

HepaRG cells and HepaRG knockout cells were treated for 24 hours in 6 well plates with the test substances. RNA from cells as well as from rat liver tissues frozen in nitrogen was isolated using peqGOLD TriFast (peqlab, Erlangen, Germany) or TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturers' protocol. Quality and quantity of the isolated RNA were controlled with a Nanodrop spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Waltham, MA, USA). If necessary, RNA was further purified using a RNA purification kit (Qiagen, Hilden, Germany).The human microarray Agilent Expression Profiling Service (incl. 8x60K Array) was performed by ATLAS Biolabs GmbH. Obtained results were further analyzed using the bioinformatic analysis and search tool IPA (Ingenuity Pathway Analysis) from QIAGEN. Therefore, the IPA "Tox Analysis" tool was used. All analyses were performed with the standard settings, where no filtering was applied and direct as well as indirect relationships were considered (date of the search: 2017/11/10). Real-time RT-PCR was performed as described previously (Heise et al., 2015). In brief, reverse transcription of 1 µg of RNA (HepaRG cells) or 2 µg RNA (rat livers) was conducted using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany). Quantitative real-time PCR was performed of 40 ng cDNA on an ABI 7900HT instrument (Applied Biosystems) using Maxima SYBR Green/ROX qPCR Mastermix (Thermo Fisher Scientific) and primers (0.25 µM, synthesized at Eurofins Genomics, see Table 2).

Table 2: Sequences of primers used for RT-PCR analysis.

gene name	forward primer	reverse primer
hGAPDH	CCACTCCTCCACCTTTGAC	ACCCTGTTGCTGTAGCCA
hACTB	ACCGAGCGCGGCTACAG	CTTAATGTCACGCACGATTTCC
hCYP1A1	TGTCAGTGGCCAACGTCATT	AGGGTTAGGCAGGTAGCGAA
hCYP1A2	TGCAAGACAAGCTGGTGTCTA	TCTCATGCGCTCACAGAACT
rGapdh	CCGTGGGGCAGCCCAGAAC	GCCCCAGCATCAAAGGTGGAGGA
rActb	AGGGAAATCGTGCGTGAC	CGCTCATTGCCGATAGTG
rCyp1a1	TTCACCATCCCCCACAGCACCATA	CAGGCCGGAACTCGTTTGGATCAC

rCyp1a2	CGGTGATTGGCAGAGATCGG	GTCCCTCGTTGTGCTGTGG

2.8 CYP protein quantification

HepaRG cells were treated with the test substance Pi for 24 hours in 6-well plates. After two washing steps with ice-cold PBS-buffer, the cells were incubated for 1 hour at 4 °C with lysis buffer under shaking (Zahn et al., 2017). Afterwards, each sample was prepared and measured as described previously (Marx-Stoelting et al., 2017; Wegler et al., 2017). In brief, a proteolysis was performed and multi-specific antibodies recognizing short C-terminal sequences of proteotypic peptide fragments were mixed with isotopically labeled peptides. With the help of an automated immunoprecipitation procedure endogenous peptides and standards for the proteins were enriched (Weiß et al., 2015). Peptide-antibody-complexes were precipitated with magnetic protein G microspheres using a magnetic particle processor. Elution was achieved with 1% formic acid and peptides were subsequently desalted by a PepMap100 µ-precolumn (0.3 mm I.D. x 5 mm, Thermo Fisher Scientific, Waltham, MA, USA) and separated by an Acclaim Rapid Separation LC (RSLC) Column (75 µm I.D. x 150 mm; Thermo Fisher Scientific, Waltham, MA, USA) on a UltiMate 3000 RSLCnano LC system (Thermo Fisher Scientific, Waltham, MA, USA). After that, peptides were quantified with a Q Exactive™ Plus (Thermo Fisher Scientific, Waltham, MA, USA) using targeted Single-Ion-Monitoring (tSIM). Ion chromatograms were processed with Skyline 3.7.0.11317 (MacCoss Lab. Department of Genome Sciences, UW, USA). Concentrations were calculated by the peak area ratios of isotopically labeled and endogenous peptides on parent ion level. The analyte-dependent lower limits of quantification (LLOQ) were determined with a dilution series of standard peptides (0.1-0.4 fmol/µg).

2.9 CYP activity assay

HepaRG cells were treated for 24 hours in 96-well plates with the test substances. 10 µM of the substrate 7-ethoxyresorufin was added to each well and incubated for 30 minutes at 37°C. Liver microsomes were isolated in a 250 mM sucrose buffer (Merck, Darmstadt, Germany) by differential centrifugation at a final speed of 100,000g for 1 h. Afterwards, the

O-dealkylation of the substrates 7-ethoxyresorufin (EROD) and 7-methoxyresorufin (MROD) were measured to detect the enzyme activity of CYP1A1/Cyp1a1 and CYP1A2/Cyp1a2 using resorufin as a standard (reagents obtained from Sigma-Aldrich, Basel, Switzerland). The assay was performed at 37 °C in a KH2PO4/K2HPO4 buffer at pH 7.4. Fluorescence measurements (535 nm/ 590 nm) were conducted on a Tecan Plate Reader (Tecan, Infinite M200Pro).

2.10 Statistical analysis

The statistical analysis was performed using SigmaPlot for Windows software (Version 13.0, Systat Software Inc. 2008). The Shapiro-Wilks and Brown-Forsythe tests were used to analyze the data for normal distribution and for homogeneity of variances. These results demonstrated that non-parametric testing is the adequate testing method for the present data sets. Therefore the Mann-Whitney rank sum test was executed to compare the solvent control to respective treatment groups. Error bars depict the standard deviation and asterisks (*) define statistical significance if $p \leq 0.05$. For the combination experiments, a statistical dose-response modeling was performed. PROAST software (www.proast.nl) was used to analyze concentration addition of the combinations, as described previously (Kienhuis et al., 2015).

3. Results

3.1 Microarray gene expression data indicate AHR activation by Pi in human cells

In the course of a study aimed to investigate global transcriptomic responses of human liver cells to the fungicidal active substance Pi *in vitro*, we discovered that *CYP1A1* and *CYP1A2*, the most classic target genes of the nuclear receptor AHR, were among the top upregulated genes in HepaRG hepatocarcinoma cells (see supplementary Table 1 for the full list of Pi-regulated genes). Microarray analysis was verified by conventional real-time RT-PCR of *CYP1A1* and *CYP1A2* (see 3.3 below). The literature-based bioinformatic IPA (Ingenuity Pathway Analysis) was used to unravel possible further hints for AHR activation by Pi. These

analyses revealed that other genes which have been previously linked to AHR activation were also altered by Pi (Figure 1A). For most of these genes the direction of regulation was in line with previously published data on AHR-dependent activation or inhibition of these genes (Figure 1A). As the results from bioinformatic data mining indicated a possible involvement of the AHR in Pi-induced effects in human liver cells, further experimentation was conducted for verification.

3.2 *In silico* docking of Pi to the AHR

In a first step molecular docking simulations were performed to assess *in silico* whether Pi can bind the ligand binding domain of human AHR. A negative ∆G value of -3.8 kcal/mol was predicted (Figure 1B), suggesting that an association between the AHR and Pi is favorable from an energetic point of view. TCDD, a reference ligand for AHR, showed a ΔG value of -5.0 kcal/mol (Figure 1C). This can still be regarded as not a so favorable value, indicating that the human AHR ligand binding domain interacts rather weakly with its reference ligand. The superposition of TCDD and Pi in the AHR ligand binding site showed a partial overlap of the aromatic rings of these two chemicals (Figure 1D). The predicted ∆G value is suggestive but not sufficiently negative to firmly support binding of Pi on AHR, just basing on our *in silico* data. For this reason, AHR activation by the compound was further studied in more detail using experimental approaches.

3.3 Pi induces AHR-dependent transcription, target mRNA, protein amount, and enzyme activity

In a next step we therefore analyzed whether Pi would activate AHR-dependent transcription using a luciferase reporter system driven by either a 1.2 kbp fragment of the human *CYP1A1* promoter, a similar *CYP1A1* promoter-derived luciferase reporter lacking the four functional AHR binding sites (“-CDEF”), or an artificial promoter construct consisting of three AHR-responsive DRE sequences (“3xDRE”). As shown in Figure 2A and 2C, Pi dose-dependently induced luciferase reporter activities from the *CYP1A1* promoter and 3xDRE constructs in

HepG2 human hepatoma cells with statistical significance, whereas no induction was visible with the AHR binding site-deficient mutant (-CDEF) reporter (Figure 2B). When compared to the positive control, the aromatic hydrocarbon and known AHR inducer BbF, it became obvious that Pi showed an onset of reporter gene activation only at substantially higher concentrations than needed for BbF to produce a response. Thus, Pi constitutes a substantially weaker AHR activator than BbF. Analyses of Pi-induced effects at higher concentrations than 40 µM, in order to add information to the right side of the dose-response-curve, were hampered by cytotoxic effects occurring at these concentrations; please see Supplementary Figure 1 for cell viability analyses of Pi in HepG2 (A) and HepaRG (B) cells. To exclude that the observed AHR activation was caused by impurities potentially present in technical grade Pi the experiments were repeated with analytical grade Pi and the results were reproduced (Supplementary Figure 2A and 2B).

Dose-dependent induction of AHR activity was subsequently confirmed by real-time RT-PCR analyses of the model AHR target genes *CYP1A1* and *CYP1A2* in HepaRG cells (Figures 3A and 3B). These results well resembled the findings at the reporter gene level. Expression of the AHR mRNA itself was not altered (data not shown). In addition, abundance of CYP1A1 was quantified at the protein level using a mass spectrometry-based approach. Results of these analyses showed Pi-dependent induction of CYP1A1 protein (Figure 3C), consistent with the findings presented above. To further substantiate our findings we studied CYP1A1-dependent catalytic activities using the classic EROD (ethoxyresorufin-O-deethylase) assay for CYP1A1 activity. Figure 3D demonstrates that Pi was able to significantly induce CYP1A1-dependent enzymatic substrate conversion in human HepaRG cells in a dose-dependent manner.

Similar effects were observed in rat liver: *Cyp1a1* and *Cyp1a2* gene expression was significantly increased at the top dose level in Pi-treated rats (Figure 4A and 4B). Additionally Cyp1a1 and Cyp1a2 enzyme activities were significantly increased in a dose-dependent manner (Figure 4C and 4D).

3.4 Effects of Pi in AHR KO, PXR KO and CAR KO HepaRG cells

Three HepaRG cell lines deficient of either AHR, PXR or CAR were treated with Pi and *CYP1A1* and *CYP1A2* gene expression was analyzed to investigate if Pi-induced gene expression was mediated by either of the three receptors. Involvement of CAR and PXR in Pi-dependent gene regulation has been reported previously. As shown in Figure 5A and 5B, *CYP1A1* and *CYP1A2* upregulation was completely blocked in AHR KO cells. Comparable results were seen when using HepG2 cells treated with a pharmacological inhibitor of the AHR and a Cyp1A1 promoter driven reporter (5C). In contrast *CYP1A1* and *CYP1A2* gene expression was upregulated in a concentration dependent manner in both PXR KO (5D and 5E) cells as well as CAR KO (5F and 5G) cells, similar to wildtype cells. These results indicate that knockout of PXR and CAR has no remarkable influence on the upregulation of these genes by Pi, whereas presence of the AHR is crucial.

3.5 Combination effects of Pi and the model AHR ligand BbF

According to a previous publication linking Pi and rodent AHR, Pi alone activates the rodent AHR but might inhibit the response to model AHR ligands (Ghisari et al., 2015). We therefore exposed human hepatic cells *in vitro* to mixtures of Pi and BbF, a model AHR activator routinely used in our laboratory. Figure 6A demonstrates that no inhibition of BbF-mediated induction of AHR-dependent 3xDRE reporter activities was observed when applied in combination with Pi. Instead, data were consistent with the assumption of concentration addition (Supplementary Figure 3A). When analyzing mRNA induction of *CYP1A1* (6B) and *CYP1A2* (6C) in response to Pi and BbF mixtures, concentration addition was observed (Supplementary Figure 3B and 3C). The analysis of respective enzyme activity of CYP1A1 in the EROD-Assay showed similar results (Figure 6D and Supplementary Figure 3D).

4. Discussion

Data presented in this paper clearly demonstrate that Pi is an activator of the human AHR, as consistently shown by analyses at the reporter gene, mRNA expression, protein abundance, and enzyme activity levels in HepG2, HepaRG, or HepaRG variants bearing knockouts of individual nuclear receptors. Furthermore, investigation of gene expression and enzyme activity in rat livers showed induction of the AHR targets Cyp1a1 and Cyp1a2 in vivo.

So far, only few previous publications have established indirect links between Pi and the AHR. (Sun et al., 2005) administered Pi to rats and observed an induction of *Cyp1a2* gene expression and resulting enzyme activity without analyzing receptor binding and transactivation. (Li et al., 2013) measured induced hepatic EROD activity in juvenile rainbow trout exposed to Pi when compared to the control group. They suggest that Pi had toxic effects in the fish through binding to the aryl hydrocarbon receptor (AHR) without analyzing receptor binding or transactivation. In human intestinal cells, an increase of CYP1A1 activity after treatment with imazalil and other conazoles like Pi has been published (Sergent et al., 2009). However, imazalil was shown not to bind AHR while Pi was not investigated. (Ghisari et al., 2015) have screened various compounds for activation of rodent AHR using a murine cell line stably transfected with an AHR-responsive reporter construct and reported a concentration of 10 µM Pi as the lowest dose to exert an effect. Given the differences in incubation conditions and reporter system used, these results appear well in line with our present findings, indicating that Pi is a weak agonist of human as well as rodent AHR. Altogether, the present data on AHR activation by Pi go far beyond what has been published previously by (Ghisari et al., 2015) who followed a screening approach in a rodent cell system rather than systematically characterizing the properties of an individual compound in human hepatic cells, as was the focus of our study.

While the AHR has initially been believed to become activated rather specifically by polycyclic aromatic compounds, such as (halogenated) polycyclic aromatic hydrocarbons, dioxins, and certain polyhalogenated biphenyls, more recent data have revealed that

numerous other compounds not exactly fitting into this class of molecules are capable of activating the receptor. For example, certain experimentally used ATP-competitive kinase inhibitors such as U0126 and SB216763 (Andrieux et al., 2004; Braeuning and Buchmann, 2009) phytochemicals such as isoflavones (Van der Heiden et al., 2009) and also endogenous metabolites such as kynurenine (Opitz et al., 2011) are activators of the AHR, which, however, in most cases do not share the potency or the long biological half-life of the model activator TCDD. The present observations thus fit well into this picture of a somewhat promiscuous receptor. Since species-specific differences with regard to the agonistic properties of foreign compounds at different nuclear receptors have been noted it is important to show activation of the human receptor to draw a conclusion to the relevance for humans. A very prominent example of species-species differences is the receptor CAR, where CITCO is considered a specific agonist of human CAR, whereas TCPOBOP activates the receptor in mice but not in humans (Tzameli et al., 2000). However, such observations are not limited to CAR: the best-studied AHR activators, e.g. TCDD or polycyclic aromatic hydrocarbons, generally activate both, the AHR of humans and of rodents. They do so, however, with sometimes remarkable quantitative differences: for example, binding of TCDD to the human AHR has been reported to occur with approximately 10-fold lower affinity than for the most common AHR variants expressed in the mouse (Ema et al., 1994). Similar differences have been observed for other AHR ligands and also for species other than only humans and rodents, as for example documented in detail in (Denison et al., 2011; Flaveny et al., 2009). Species differences have also been described with respect to AHR antagonists, for example SR1 which acts as an antagonist of human but not rat AHR (Boitano et al., 2010). These observations underline the importance of conducting studies with human cells in order to assess possible toxicological consequences of nuclear receptor activation by foreign compounds in humans. It has to be noted that comparative analyses of the response of the AHRs from different species are not routinely performed, as can be taken from the lack of such data in many publications identifying novel AHR agonists.

In the paper by (Ghisari et al., 2015) it is mentioned that Pi attenuated the AHR-inducing effects of the model AHR activator TCDD in the murine cell system used there. As, however, no exact values are given, one cannot judge the strength and/or significance of this observation. Due to the fact that toxicological effects of mixtures have become an increasingly important topic during the past years, we investigated how Pi and the model AHR ligand BbF jointly influence AHR activity in human cells. Our results do not support the abovementioned finding by (Ghisari et al., 2015) according to which one would expect AHR activation by a model ligand to be diminished by Pi. Instead, our findings are in line with a model of concentration addition. The validity of this model for AHR activators constitutes the basis of current assessment of classic AHR activators: for these substances, additivity is assumed and concentrations of individual substances, multiplied with their equivalence factor (a measure for their potency relative to TCDD), can be added in order to predict a mixture effect. Our present data suggest that this type of additivity is also valid for mixtures of Pi with other AHR activators.

Pi belongs to a large family of frequently used fungicidal active substances, the triazole fungicides. These compounds are closely related with respect to their molecular structure. The finding that Pi is an AHR activator thus triggers the question whether this holds also true for other members from this chemical class. We have investigated two related substances, namely cyproconazole and tebuconazole. We couldn't observe any AHR activation after treatment with cyproconazole or tebuconazole when tested with the sensitive endpoint of luciferase reporter analysis up to the highest possible non-cytotoxic concentrations (own unpublished data). Instead, cyproconazole acts as an activator of human and rodent PXR and also rodent but not human CAR (Marx-Stoelting et al., 2017), whereas tebuconazole also activates PXR but is an antagonist of CAR (Knebel et al., 2018). Together these observations demonstrate that despite evident similarities at the level of chemical structure, the molecular targets of these substances differ considerably. This might have implications for risk assessment, where adverse effects in the liver following activation of certain nuclear receptors by xenobiotics is not considered relevant for humans, as in case of the peroxisome

proliferator-activated receptor (PPAR) alpha (Corton et al., 2014), or is subject of a controversial scientific debate, as in case of CAR activation (Braeuning et al., 2014; Braeuning et al., 2016; Braeuning et al., 2015; Elcombe et al., 2014; Yamada et al., 2014). By contrast, the most potent and specific xenobiotic inducer of the AHR, TCDD, has been classified as carcinogenic to humans by the International Agency for the Research on Cancer (IARC) and others (Baan et al., 2009). Different triazole fungicides appear to activate different sets of nuclear receptors and show variable potencies at the individual receptors. Adverse liver effects in experimental animals exposed to such substances might be contributed to by a single receptor or by a combination of multiple receptors simultaneously activated by a certain compound. Based on classic histopathology it appears almost impossible to deduce a specific molecular mechanism from observations at the tissue level. Many adverse outcome pathways (AOP) describing adversity as a sequence of molecular events triggering an adverse outcome at the organ level contain multiple molecular initiating events which later converge at the cellular level (www.aopwiki.org). For example, the AOP for liver steatosis comprises almost all important nuclear xeno-sensing receptors, such as AHR, CAR, PXR, and PPAR. Proof of activation of a certain receptor by a chemical does not always allow drawing conclusions on the prevailing molecular mechanisms, since a compound, e.g. a triazole, might be able to simultaneously act on a whole set of receptors. Thus, when judging on the human relevance of experimental data, not only possible species differences with respect to ligand-receptor interaction should be taken into account, but it should also be regarded as important to keep in mind that mechanistic toxicological argumentation for or against human relevance of a certain adverse effect should involve the different possible modes of action which are driven by individual nuclear receptors but lead to a similar adverse outcome.

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Figures

Figure 1: Bioinformatic data mining and in silico modeling indicate a possible interaction of propiconazole (Pi) with the AHR. (A) Bioinformatic analysis of microarray gene expression data with IPA indicates AHR activation by propiconazole (Pi) in HepaRG cells. The global transcriptomic responses of HepaRG cells to 10 µM Pi were analyzed with the help of the microarray Agilent Expression Profiling Service. Ingenuity Pathway Analysis (IPA) of the data indicates that classic target genes of the nuclear receptor AHR were up (red)- or downregulated (green). Duplicates gene identifiers marked with an asterisk (*) indicate that multiple identifiers in the dataset file map to a single gene in the Global Molecular Network. Colors of the connections represent the predicted relationships (activation (orange), inhibition (blue), findings inconsistent with state of downstream molecule (yellow), and effects not predicted (gray)). *In silico* molecular docking analyses of Pi to the AHR ligand binding domain are shown as graphic representations of the top-scoring complexes between Pi and AHR (B), the reference AHR ligand TCDD and AHR (C), and a superposition of Pi and TCDD (D).

Figure 2: Propiconazole (Pi) induces AHR-dependent transcription in HepG2 cells. (A) Dose-dependent induction of a luciferase reporter system driven by a 1.2 kbp fragment of the human *CYP1A1* promoter. (B) By contrast, no induction is measurable with a similar reporter lacking the four functional AHR binding sites ("-CDEF"). (C) Pi also leads to a dose-dependent induction of the artificial promoter construct consisting of three AHR-responsive DRE sequences ("3xDRE"). Induction by the model AHR ligand BbF is shown for comparison. Fold induction above solvent control (0.2% DMSO) was calculated. Mean ± SD (n=3 independent experiments, each in 3 replicates) is depicted. Statistical significance ($p<0.05$) is indicated by asterisks.

Figure 3: Propiconazole (Pi) is an agonist of human AHR. Induction of *CYP1A1* (A) and *CYP1A2* (B) mRNA levels in HepaRG human hepatoma cells by Pi, as measured by quantitative real-time RT-PCR. (C) Pi-dependent induction of CYP1A1 protein level was observed by using a mass spectrometry-based approach. (D) Induction of CYP1A1 enzyme activity by Pi, as measured by the EROD assay. Fold induction above solvent control (0.2% DMSO) was calculated. Mean ± SD ($n\geq3$) is depicted. Statistical significance ($p<0.05$) is indicated by asterisks. No statistical significance was calculated for the data in panel (C), due to the fact that these values were not derived from entirely independent experiments.

Figure 4: Propiconazole (Pi) induces AHR target genes and resulting enzyme activities in rat livers. Pi leads to an induction of *Cyp1a1* (A) and *Cyp1a2* (B) mRNA levels in rat livers as well as to an induction of the enzyme activities of Cyp1a1 (C) and Cyp1a2 (D). Figures show fold induction relative to the respective controls. Mean ± SD ($n_{biological} = 5$) is depicted. Statistical significance ($p<0.05$) is indicated by asterisks.

Figure 5: Upregulation of AHR target genes was blocked in AHR KO cells. No induction of *CYP1A1* (A) and *CYP1A2* (B) gene expression was measured in AHR KO cells Additionally

no transactivation of Cyp1A1 promoter was measured with 10µM of the AHR inhibitor CH223191 (C). In contrast, investigation of *CYP1A1* and *CYP1A2* in PXR KO (D, E) and CAR KO (F, G) cells showed concentration-dependent upregulation, indicating that the latter two nuclear receptors are not involved in Cyp1a1/2 induction by propiconazole. Fold induction above solvent control (0.2% DMSO) was calculated. Mean ± SD (n≥3) is depicted. Statistical significance ($p<0.05$) is indicated by asterisks. For the receptor experiments, no statistical significance was calculated, due to the fact that these values were not derived from entirely independent experiments.

Figure 6: Propiconazole (Pi) and the more potent model ligand benzo[*b*]fluoranthene (BbF) have concentration additive effects on human AHR. (A) Pi and BbF both lead to a dose-dependent induction of the artificial 3xDRE promoter construct. Mixtures of both compounds suggest concentration additive combination effects on AHR activation. Furthermore, induction of *CYP1A1* (B) and *CYP1A2* (C) mRNA levels in human HepaRG cells by Pi and BbF shows concentration additivity. (D) The results from the EROD assay, which shows the induction of CYP1A1 enzyme activity by Pi and BbF, are also in line with concentration additivity. Fold induction above solvent control (0.2% DMSO) was calculated. Mean ± SD (n=3 independent experiments, each in 3 replicates) is depicted. Statistical significance ($p<0.05$) is indicated by asterisks.