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The azole fungicide tebuconazole affects human *CYP1A1* and *CYP1A2* expression by an aryl hydrocarbon receptor-dependent pathway

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ABSTRACT

Tebuconazole, a member of the triazole group of fungicides, exerts hepatotoxicity in rodent studies. Knowledge on the molecular mechanisms underlying tebuconazole toxicity is limited. Previous studies suggest that activation of xenobiotic-sensing nuclear receptors plays a role in triazole fungicide-mediated hepatotoxicity. This study aimed to characterize the ability of tebuconazole to activate gene expression via the aryl hydrocarbon receptor (AHR). Results demonstrate a statistically significant induction of the AHR target genes *CYP1A1* and *CYP1A2* in HepG2 and HepaRG human liver cells *in vitro* at concentrations corresponding to tebuconazole tissue levels reached under subtoxic conditions *in vivo*. *CYP1A1* and *CYP1A2* induction was abolished in the presence of an AHR antagonist or in AHR-knockout HepaRG cells, substantiating the importance of the AHR for the observed effects. Although the results indicate that tebuconazole is a weak inducer of AHR-dependent genes, combined exposure of HepaRG cells to tebuconazole and the previously identified AHR agonist propiconazole showed additive effects on *CYP1A1* and *CYP1A2* expression. In summary, we demonstrate that AHR-downstream gene expression is affected by tebuconazole in an AHR-dependent manner. Data indicate that dose addition may be assumed for the assessment of AHR-related effects of triazole fungicide mixtures.

1. Introduction

Triazole fungicides are used in agriculture and in human and veterinary medicine to treat a broad spectrum of fungal disease. They are designed to inhibit fungal cytochrome P450 (CYP) 51 (lanosterol-14α-demethylase) to block cell membrane synthesis (Georgopapadakou, 1998). As a side effect they may inhibit mammalian CYP enzymes in an unspecific manner, among them are also some CYPs essential for synthesis of steroid hormones (e.g. aromatase, CYP19A1) or for the metabolism of vitamins such as vitamin A (Robinson et al., 2012; Vinggaard et al., 2006; Zarn et al., 2003). It is therefore not surprising that triazole fungicides may cause adverse effects related to this mode of action. In studies conducted for the regulatory approval of tebuconazole as an active substance for pesticidal use, for example, the compound caused effects in steroidogenic endocrine organs and developmental toxicity. Tebuconazole induced hypertrophy of adrenal glands in oral short-term toxicity studies in rats and dogs, and was also classified for developmental toxicity (H361d) due to malformations observed in several species (EFSA, 2014).

In general, however, the most sensitive target organ of triazoles is the liver. This is, at least in part, attributed to the fact that these substances also interact with hepatic nuclear receptors such as the constitutive androstane receptor (CAR) (Goetz and Dix, 2009). Hence it is not surprising that recently tebuconazole was also shown to cause effects on the liver *in vivo* (EFSA, 2014; Schmidt et al., 2016). In short term studies tebuconazole exposure led to liver weight increase and centrilobular hypertrophy in rats and mice (EFSA, 2014; Schmidt et al., 2016). In mice but not in rats tebuconazole additionally caused

Abbreviations: AHR, aryl hydrocarbon receptor; CAR, constitutive androstane receptor; CYP, cytochrome P450; DMSO, dimethyl sulfoxide; DRE, dioxin response element; EROD/MROD, 7-ethoxy/methoxyresorufin-O-deethylase; FCS, fetal calf serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PXR, pregnane-X-receptor

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hepatocellular tumors in long-term studies, which was not considered relevant for classification due to assumed species differences between mice and men (EFSA, 2014).

Indeed, triazole-induced liver effects may exhibit significant species differences. For example, Marx-Stoelting et al. (2017) showed that hepatic responses to another azole fungicide (cyproconazole) were remarkably less pronounced in mice with humanized CAR and pregnane-X-receptor (PXR) as compared to wild-type controls, suggesting that the compound was not able to stimulate the receptors from the two species to a comparable degree. Species differences between rodents and humans with regard to the hepatic consequences of CAR activation, especially tumor findings in animal studies, are controversially discussed among toxicologists (Braeuning, 2014; Braeuning et al., 2015; Braeuning and Schwarz, 2016; Elcombe et al., 2014; Yamada et al., 2015).

Tamura et al. (2013, 2015) suggested that activation of CAR may not be responsible for tebuconazole-dependent liver effects observed in mice. Furthermore, in a recent study it was shown that tebuconazole is not an agonist but an antagonist of human and rat CAR (Knebel et al., 2018b). Hence, tebuconazole-mediated toxicity most likely cannot be attributed to activation of CAR alone, but other hepatic nuclear receptors have to be considered. Indeed, data from global gene expression analysis of some azole fungicides suggest the involvement of other nuclear receptors than just CAR in azole-mediated liver toxicity (e.g. see Goetz and Dix (2009) and Heise et al. (2015)). Among them are PXR (activation by tebuconazole already analyzed by Knebel et al. (2018b)) and the aryl hydrocarbon receptor (AHR). Since other structurally related substances like prochloraz or propiconazole have already been shown to activate AHR (Halwachs et al., 2013; Knebel et al., 2018a; Rieke et al., 2014) and tebuconazole has been shown to activate CYP1A1 and Cyp1a1/2 activity in EROD/MROD (7-ethoxy/methoxyresorufin-O deethylase) assays (Sergent et al., 2009; Yang et al., 2018) it is reasonable to test the potential of tebuconazole to activate human and murine AHR.

In this study we therefore investigated the potential of tebuconazole to activate the AHR *in vivo* and *in vitro*, in rat liver and in human cell lines of hepatic origin.

2. Materials and methods

2.1. Test compounds

Technical grade tebuconazole (CAS no. 107534-96-3; Batch no. NK21BX0392; purity 96.20%) as applied in plant protection products was obtained from Bayer (Leverkusen, Germany). Analytical grade tebuconazole (CAS no. 107534-96-3; Batch no. G142375; purity 99.30%) was purchased from LGC Standards (Wesel, Germany). The AHR inhibitor CH-223191 (CAS no. 301326-22-7; Batch no. 12131228) was purchased from Sigma-Aldrich (Taufkirchen, Germany) and the AHR agonist 3-methylcholanthrene (CAS no. 56-49-5; purity 97%) from Enzo life sciences (Lörrach, Germany). Technical grade propiconazole (CAS no. 60207-90-1; Batch no. CGA64250B; purity 96.10%) was obtained from Syngenta (Basel, Switzerland). All test substances were dissolved in dimethylsulfoxide (DMSO) to result in a final solvent concentration of 0.2% (v/v) in cell culture medium.

2.2. Material from animal studies

Liver samples from a previous study (Schmidt et al., 2016) were used. In that study, male Wistar rats had been treated with dose levels of 1, 10, or 1000 ppm of tebuconazole, added to a phytoestrogen-free rodent standard diet (R/M-H V155; Ssniff, Soest, Germany), for 28 consecutive days starting at 5–6 weeks of age. All animal received humane care and the experiment had been approved by a local ethics committee. Livers had been excised from the animals following sevoflurane anesthesia and subsequent sacrifice by 95% CO_2/5% O_2. Samples had been directly frozen on dry ice for future analysis. Standard histopathological examination revealed a 7% increase in relative liver weights by the highest dose of tebuconazole along with centrilobular hypertrophy, but no adverse effects (Schmidt et al., 2016). Tebuconazole tissue levels after exposure to 1000 ppm of the compound have been determined according to the ASU L 00.00–115 LC method (Schmidt et al., 2016).

Independent from the above animal study, S9 fractions were generated from the livers of rats induced with phenobarbital and β-naphthoflavone following standard methodology according to OECD guideline 471. The composition of the S9 mix consisted of 33 mM potassium chloride, 8 mM magnesium chloride, 4 mM nicotinamide adenine dinucleotide phosphate, 5 mM glucose-6-phosphate and 10% induced rat S9 fraction diluted in 15 mM sodium phosphate buffer (pH 7.4).

2.3. Cell cultivation and treatment

HepG2 cells (ECACC, Porton Down, UK) were incubated in DMEM medium (Pan-Biotech, Aidenbach, Germany) which was supplemented with 10% fetal calf serum (FCS) (Pan-Biotech). When the confluency of the cells was about 80%, cells were passaged and used until passage 12. Phenol red-free DMEM medium (Pan-Biotech) supplemented with 10% FCS was used for the treatment of the cells. HepG2 cells were treated for a period of 24 h and used for cytotoxicity testing and reporter gene assays.

For the experiments with the rat S9 mix, tebuconazole and 20% S9 mix were preincubated for 30 min at 37 °C. Afterwards, HepG2 cells were treated with the preincubated tebuconazole for 4 h. In the next step, treatment medium was removed, fresh medium was added and cells were incubated for additional 20 h before the reporter gene assays were performed.

HepaRG cells (Biopredic International, Saint Grégoire, France) were cultured as previously described (Gripon et al., 2002; Knebel et al., 2018b). In brief, cells were incubated for two weeks in proliferation medium (Williams E medium (Pan-Biotech), 10% FCS (Pan-Biotech), 100 U/ml penicillin, 100 µg/mL streptomycin, 0.05% human insulin (all from PAA Laboratories GmbH, Pasching, Austria) and 50 µM hydrocortisone-hemisuccinate (Sigma-Aldrich)). Afterwards, cells were differentiated for two weeks in differentiation medium (proliferation medium supplemented with 1.7% DMSO). After differentiation, cells were treated with test substances in treatment medium (phenol red-free Williams E medium (Pan-Biotech) supplemented like the differentiation medium, but only 2% FCS and 0.5% DMSO).

Different HepaRG sub-lines with knockout of individual xeno-sensing receptors were also cultured as previously described (Knebel et al., 2018a) and according to the manufacturers' protocol. In brief, AHR, PXR, and CAR knockout HepaRG cells (Sigma-Aldrich) were thawed and cultivated for two days in recovery medium (Sigma-Aldrich). For the following two weeks, cells were cultured in maintenance medium (Sigma-Aldrich). Afterwards, pre-induction-medium (Sigma-Aldrich) was added to the cells for one day. Treatment with the test substances was performed in serum-free induction medium (Sigma-Aldrich).

All cells were incubated at 37 °C and 5% CO_2 in a humidified atmosphere in a Binder cell culture incubator.

2.4. Cytotoxicity tests

Cytotoxicity induced by the treatment with the test substances was analyzed by performing the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay in HepG2 and HepaRG cells according to standard protocols (Braeuning et al., 2012). In brief, 40,000 cells per well (HepG2) or 9000 cells per well (HepaRG) were seeded in 96-well plates. Following incubation of cells with test compounds, MTT reagent was added for an incubation time of 1 h. Afterwards, cells were centrifuged and lysed in desorption solution (0.7%

sodium dodecylsulfate in isopropanol) for another 30 min. Finally, absorption was measured at a wavelength of 570 nm with a reference wavelength of 630 nm using a plate reader (Infinite M200PRO, Tecan, Männedorf, Switzerland).

2.5. RNA isolation and gene expression analysis

Rat liver tissues from the 28-day feeding study (see material from animal studies) were available as samples frozen in liquid nitrogen. For RNA isolation from cell cultures, 500,000 cells per well (HepG2) or 200,000 cells per well (HepaRG) were seeded in 6-well plates and incubated with the test substances for 24 h. RNA isolation from rat livers was performed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and RNA isolation from the cell lines using peqGOLD TriFast (peqlab, Erlangen, Germany) according to the manufacturers' protocols. Quality and quantity of the isolated RNA were controlled with a Nanodrop spectrophotometer (NanoDrop, 2000, Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription of 1 µg RNA (HepG2, HepaRG and HepaRG knockout cells) or 2 µg RNA (rat livers) was conducted with the help of the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany). Finally, real-time RT-PCR was performed as described previously (Heise et al., 2015; Knebel et al., 2018b). In brief, 40 ng cDNA was analyzed on an ABI 7900HT instrument (Applied Biosystems) with Maxima SYBR Green/ROX qPCR Mastermix (Thermo Fisher Scientific) and primers (0.25 µM, synthesized at Eurofins Genomics, see Table 1).

The human microarray Agilent Expression Profiling Service was performed by ATLAS Biolabs GmbH (Berlin, Germany). The results were further analyzed with the "Tox Analysis" tool from the bioinformatic analysis and search tool Ingenuity Pathway Analysis (IPA, Qiagen, Germantown, MD, USA). The analysis was performed with standard settings (no filtering, direct and indirect relationships were considered; date of the analysis: 2018/07/24).

2.6. CYP activity assays

The CYP enzyme activity in rat livers was measured as described elsewhere (Heise et al., 2015). Briefly, liver microsomes were isolated by several steps of ultracentrifugation at a final speed of 100,000 × *g* for 1 h. These microsomes were used to measure the O-dealkylation of the substrates 7-ethoxyresorufin (EROD; Sigma-Aldrich) and 7-methoxyresorufin (MROD; Sigma-Aldrich) to detect the enzyme activity of Cyp1a1 and Cyp1a2, respectively. The assay was performed at 37 °C in a KH_2PO_4/K_2HPO_4 buffer at pH 7.4. Fluorescence measurements (excitation: 535 nm; emission: 590 nm) were conducted on a Tecan Plate Reader (Tecan, Infinite M200Pro). Resorufin (Sigma-Aldrich) was used as a standard.

CYP1A2 activity in HepaRG cells *in vitro* was determined using a mass-spectrometric approach as previously described (Feidt et al., 2010; Knebel et al., 2018b). In brief, a CYP substrate cocktail was added to the cells 2 h prior to harvest. Following incubation cell culture supernatant aliquots were mixed with 1/10 volume of 250 mM formic acid and then used for mass-spectrometric quantification of substrate metabolites as reported in Feidt et al. (2010). In total, the following CYP substrates were present in the assay in the indicated concentrations (of which only CYP1A2 was of interest here): CYP1A2, phenacetin (50 µM); CYP2B6, bupropione (25 µM); CYP2C8, amiodaquine (5 µM); CYP2C9, tolbutamide (100 µM); CYP2C19, mephenytoin (100 µM); CYP2D6, propafenone (5 µM); CYP2E1, chlorzoxazone (50 µM); CYP3A4, midazolam (10 µM).

2.7. Transfection and luciferase reporter assay

HepG2 cells were seeded in 96-well plates (40,000 cells per well) 24 h prior to transfection with TransIT-LT1 (Mirus Bio LCC, Madison, WI, USA) as recently described (Knebel et al., 2018b). Cells were transfected for 5 h either with a pT81/luc-based reporter plasmid encoding Firefly luciferase under the control of a 1.2 kbp fragment of the human *CYP1A1* promoter which contains four functional AHR binding sites (dioxin response elements, DREs), or with a pT81/luc-based reporter plasmid encoding Firefly luciferase under the control of an artificial array of three DREs. Both plasmids have been described in detail previously (Schulthess et al., 2015). Treatment of cells with the test compounds was started 24 h after transfection for additional 24 h (3x DRE) or 48 h (*CYP1A1* promoter), as described previously (Knebel et al., 2018b). Cell treatment with 4 µM of the model agonist 3-methylcholanthrene was performed as a positive control for the assay. A plasmid encoding Renilla luciferase under the control of a constitutively active promoter was co-transfected as internal control. Following cell lysis according to an established protocol, luciferase activities were measured on a Tecan Infinite M200PRO plate reader (Tecan). Firefly luciferase signals were normalized to Renilla luciferase activities.

2.8. Data processing and statistical evaluation

To analyze potential concentration addition with a statistical dose-response modeling approach, the software PROAST (www.proast.nl) was used. This software has been described previously (Kienhuis et al., 2015). SigmaPlot for Windows software (Version 14.0) was used for figure layout and to perform statistical analyses. In a first step, the Shapiro-Wilks (normal distribution) and the Brown-Forsythe (homogeneity of variances) tests were performed to determine if the data meet the prerequisites for parametric testing. Due to the fact that most of the data were not suited for parametric testing, non-parametric testing was applied using the Mann-Whitney rank sum test. ANOVA analysis was performed for enzyme activity assays. Asterisks (*) indicate statistical significance assumed at $p < 0.05$, and error bars depict the standard deviation.

Table 1
Sequences of primers used for qPCR analysis.

Gene	Forward primer	Reverse primer
rGapdh	CCGTGGGGCAGCCCAGAAC	GCCCCAGCATCAAAGGTGGAGGA
rActb	AGGGAAATCGTGCGTGAC	CGCTCATTGCCGATAGTG
rCyp1a1	TTCACCATCCCCCACAGCACCATA	CAGGCCGGAACTCGTTTGGATCAC
rCyp1a2	CGGTGATTGGCAGAGATCGG	GTCCCTCGTTGTGCTGTGG
hGAPDH	CCACTCCTCCACCTTTGAC	ACCCTGTTGCTGTAGCCA
hACTB	ACCGAGCGCGGCTACAG	CTTAATGTCACGCACGATTTCC
hCYP1A1	TGTCAGTGGCCAACGTCATT	AGGGTTAGGCAGGTAGCGAA
hCYP1A2	TGCAAGACAAGCTGGTGTCTA	TCTCATGCGCTCACAGAACT
hUGT1A1	GTGCGACGTGGTTTATTCCC	GGCTTCAAATTCCTGGGATAGTG
hUGT1A6	CCTGGAGCATACATTCAGCAGAA	AAGGAAGTTGGCCACTCGTTG

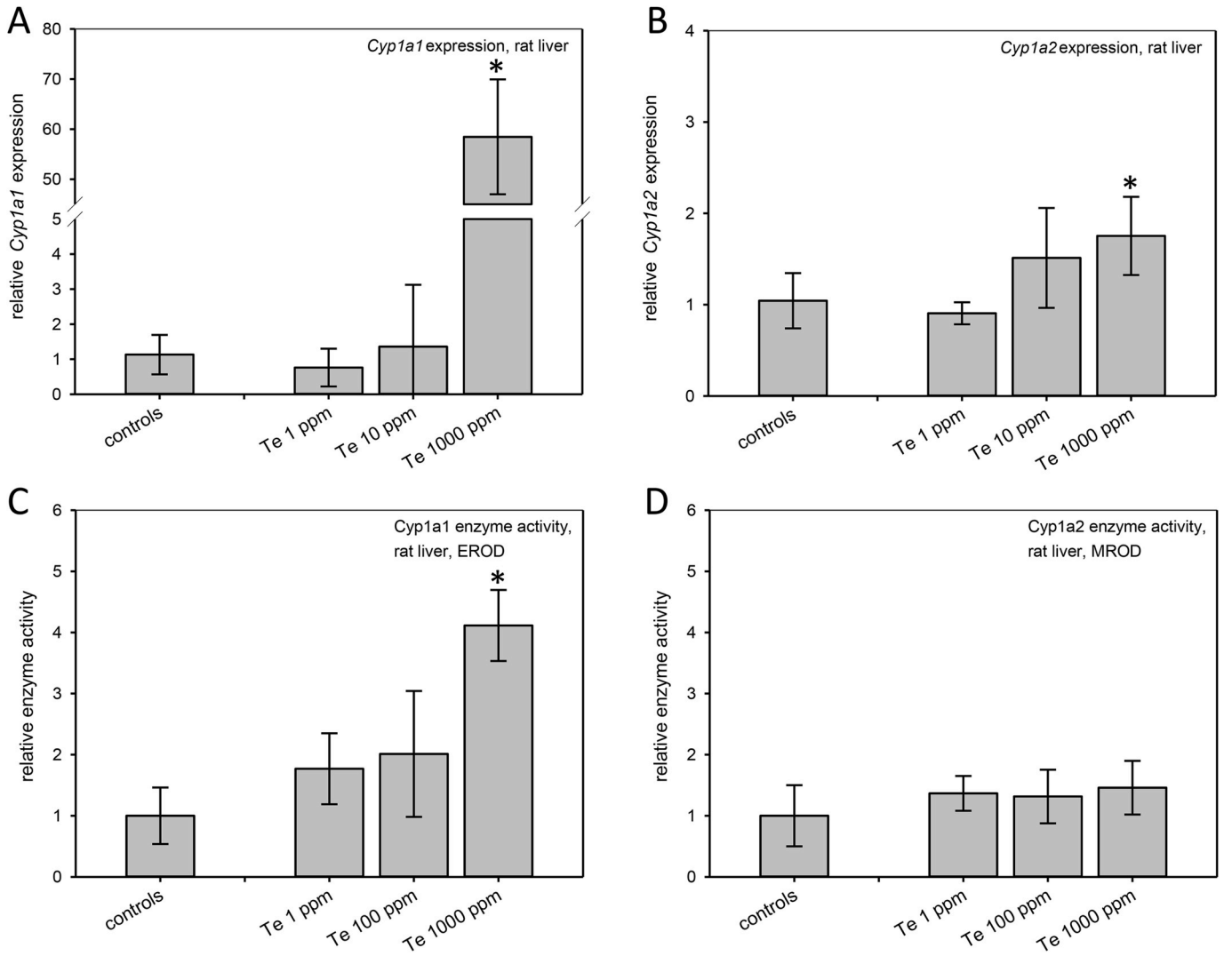

Fig. 1. Oral exposure of rats to 1, 10 or 1000 ppm tebuconazole (Te) via the diet for 28 days leads to elevated expression of AHR-dependent CYPs. Real-time RT-PCR analyses of *Cyp1a1* (A) and *Cyp1a2* (B) expression are shown relative to vehicle controls, together with enzyme activity data for Cyp1a1 (C) and Cyp1a2 (D). Mean ± SD (n = 5) are depicted. Statistical significance ($p < 0.05$ vs. controls) is indicated by asterisks.

3. Results

3.1. Tebuconazole induces Cyp1a1 and Cyp1a2 in rats in vivo

In the course of the evaluation of a previous *in vivo* study in rats exposed to up to 1000 ppm (corresponding to a mean daily intake of 71.24 (± 4.38) mg/kg bw) tebuconazole via the diet for 28 days, real-time RT-PCR analyses revealed that the highest dose of tebuconazole caused a statistically significant induction of the expression of the mRNAs encoded by the *Cyp1a1* and, to a lesser extent, *Cyp1a2* genes (Fig. 1A and B). Accordingly, enzymatic activity of Cyp1a1 was significantly up-regulated in liver tissue of tebuconazole-exposed rats (Fig. 1C), whereas no remarkable effect was detected for Cyp1a2 enzyme activity (Fig. 1D). *Cyp1a1* and *Cyp1a2* constitute the prototypical target genes of the AHR and therefore suggested that the latter receptor might be activated by tebuconazole. Tissue levels of tebuconazole in the livers from the above rat study have already been reported earlier (Schmidt et al., 2016): tebuconazole was determined in rat liver tissue at a mean concentration of 1.80 ± 0.57 µg/g tissue, corresponding to approximately 5.8 µM.

3.2. Tebuconazole induces AHR-dependent genes in human cells in vitro

We next analyzed whether tebuconazole would also affect the human AHR-dependent genes *CYP1A1* and *CYP1A2*. For this purpose, the two hepatic cell lines HepG2 and HepaRG were used. The tebuconazole concentration range for subsequent analyses was defined with the help of cytotoxicity testing (Fig. 2A and B): a concentration of 40 µM tebuconazole was chosen as the maximum concentration for further experimentation to ensure the absence of artifacts caused by cytotoxicity. The selected non-cytotoxic range included the experimentally determined *in vivo* liver concentration of tebuconazole in rats (see above).

Tebuconazole induced the mRNA levels of *CYP1A1* in both cell lines in a statistically significant and concentration-dependent manner, with more pronounced responses in HepaRG cells (Fig. 2C and D). Induction of *CYP1A2*, which was exclusively expressed in HepaRG cells, was observed similarly (Figure E). Moreover, CYP1A2 enzyme activity in HepaRG cells was determined by means of a mass-spectrometric method for multiplexed detection of CYP activities (Feidt et al., 2010; Knebel et al., 2018b). Up-regulation of CYP1A2 enzyme activity, measured as phenacetin O-deethylation, was observed in HepaRG cells, consistent with the findings at the mRNA level (Fig. 2F). In order to broaden the evidence for an activation of the AHR by tebuconazole, a

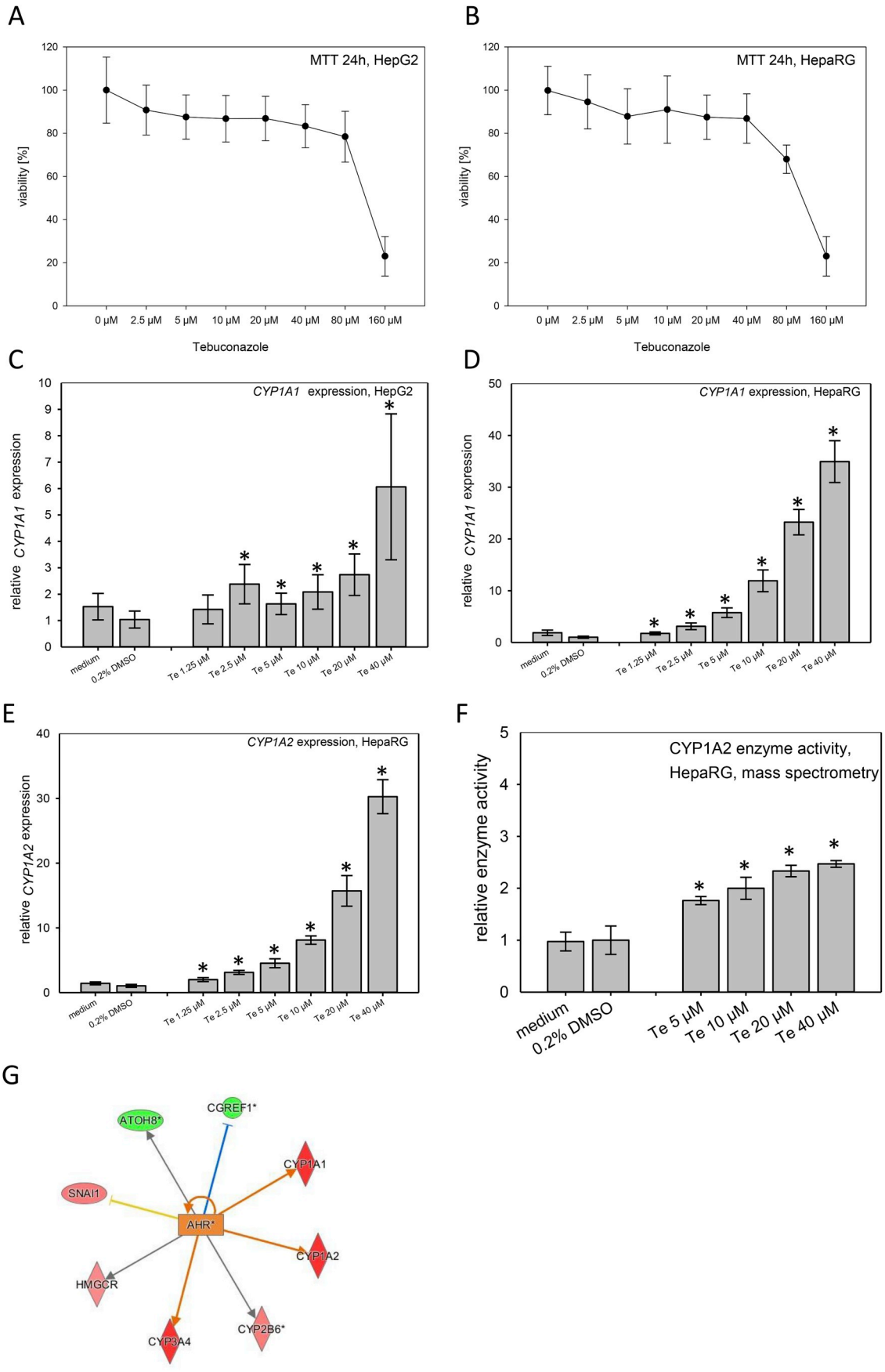

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Fig. 2. Tebuconazole (Te) induces the expression of AHR target genes in the human liver cell lines HepG2 and HepaRG. Results from initial cytotoxicity tests with tebuconazole in HepG2 (A) and HepaRG (B) cells are shown. The detergent Triton X-100 (0.01%) was used as positive control. Incubation of cells with non-cytotoxic tebuconazole concentrations for 24 h significantly induced *CYP1A1* mRNA expression in HepG2 (C) and HepaRG (D) cells, as compared to the appropriate solvent control (0.2% DMSO). A concentration-dependent and statistically significant induction of *CYP1A2* mRNA was observed in HepaRG cells (E). Accordingly, CYP1A2 enzyme activity (phenacetin O-deethylation) was significantly upregulated by tebuconazole (F). Mean ± SD (n = 3 independent experiments, each in three replicates; n = 3 without additional replicates for CYP1A2 enzyme activity analysis) are depicted. Statistical significance ($p < 0.05$) is indicated by asterisks. (G) Bioinformatic analysis of tebuconazole effects, as determined by microarray transcriptome analysis. AHR-related genes are depicted, as identified using IPA software. Color code: orange/red gene, experimentally observed upregulation; green genes, experimentally observed downregulation; orange arrows, observed upregulation is in line with AHR-dependent effects from the IPA database; blue arrows, observed downregulation is in line with AHR-dependent effects from the IPA database; gray arrows, no direction of regulation predicted by IPA; yellow arrow, observed upregulation is not in line with the IPA database. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
AHR-related gene expression changes in tebuconazole-treated HepaRG cells, as determined by IPA analysis of microarray data. Only probe sets meeting the criteria of a $|\log_2 \text{ratio}| > 1.5$ for the fold change and $p < 0.05$ for statistical significance are presented.

Gene	Fold change	p-value
ATOH8	−2.10	0.006
CGREF1	−2.12	0.044
CYP1A1	9.35	< 0.001
CYP1A2	6.82	< 0.001
CYP2B6	2.30	0.006
CYP3A4	6.12	< 0.001
HMGCR	2.11	0.007
SNAI1	2.35	0.002

genome-wide microarray gene expression analysis was conducted with RNA isolates from tebuconazole-treated HepaRG cells. The full list of differentially expressed genes is contained in Supplemental Table 1. From the microarray data set, eight genes were identified which were significantly affected by 10 μM tebuconazole and linked to the AHR using the bioinformatic data mining software IPA (Fig. 2G). The individual genes are presented along with their fold regulation and p-values in Table 2. With the exception of one gene (*SNAI1*), the observed direction of expression changes was in line with the literature-based predictions by IPA (Fig. 2G). In addition, the expression of *UGT1A1* and *UGT1A6* was analyzed in HepaRG and HepG2 cells to increase confidence in the alteration of AHR-dependent genes by tebuconazole. Observed changes were, as expected, much less pronounced than for *CYP1A1* and *CYP1A2* which generally provide much higher fold changes in response to AHR activation than the UGTs. Nonetheless, a tendency for induction was also seen for *UGT1A1* and *UGT1A6* expression, even though some differences in responsiveness were noted for the two cell lines (Supplemental Fig. 1). Taken together, the above findings demonstrate that AHR target genes are affected by tebuconazole also in human liver cells, thus suggesting that tebuconazole might act as an activator of AHR-dependent transcription.

Of note, technical grade tebuconazole as used for pesticide product formulation may contain unknown impurities which in principle might interfere with the AHR. To verify that the observed changes are indeed to be attributed to the pesticidal active compound tebuconazole, analyses of *CYP1A1* and *CYP1A2* mRNA induction in HepaRG cells were repeated with analytical grade tebuconazole. These results are presented in Supplemental Fig. 2 and demonstrate that the observed effect is similarly measured following incubation with high-purity tebuconazole.

3.3. *CYP1A1 and CYP1A2 induction by tebuconazole is dependent on the AHR*

The role of the AHR in tebuconazole-mediated *CYP1A1* and *CYP1A2* induction in HepaRG cells was verified using two different approaches: first, incubation of HepaRG cells with tebuconazole was performed in the presence of an AHR inhibitor. The induction of *CYP1A1* and *CYP1A2* mRNAs by 10 μM tebuconazole was significantly inhibited when the cells were simultaneously treated with the 10 μM of the AHR inhibitor CH-223191 (Fig. 3A and B). In a second approach, *CYP1A1* and *CYP1A2* mRNA induction by tebuconazole was abolished in a commercially available AHR knockout variant of HepaRG cells (Fig. 3C and D).

Tebuconazole is further known to interact with the nuclear receptors PXR and CAR, which also play a role in the regulation of drug-metabolizing enzymes (Knebel et al., 2018b). We therefore next analyzed whether the observed *CYP1A1* and *CYP1A2* induction by tebuconazole would depend on one of the latter receptors. For this purpose commercially available sub-lines of HepaRG with a knockout of either CAR or PXR were employed. *CYP1A1* and *CYP1A2* mRNAs were still inducible in the absence of PXR or CAR, indicating that these two receptors do not play a substantial role in tebuconazole-mediated induction of the two AHR target genes (Fig. 4).

Additional analyses were carried out using two different AHR-dependent luciferase reporter gene assays, one driven by a fragment of the human *CYP1A1* promoter, the other driven by an artificial array of three AHR binding sites, called DREs (Schulthess et al., 2015). HepG2 cells were used for this series of experiments because HepaRG cells are barely transfectable with plasmids. The positive control 3-methylcholanthrene significantly induced luciferase signals from both reporter systems, demonstrating functionality of the assay (Supplemental Fig. 3). By contrast, no increase of luciferase activities was observed following treatment with tebuconazole up to concentrations of 40 μM (Supplemental Fig. 3). Additional experiments were performed with tebuconazole in combination with an extracellular metabolic activation system (S9 mix) in order to compensate for the weak metabolic activity of HepG2 cells. Nonetheless, tebuconazole did not alter AHR-dependent luciferase activities also under these conditions (data not shown).

3.4. *Effects of mixtures of AHR-activating triazole fungicides follow dose addition*

AHR activation by propiconazole, a structurally related fungicide, has been reported recently (Knebel et al., 2018a). Simultaneous exposure to different triazole-class fungicides is a realistic scenario, and therefore the effects of mixtures of propiconazole and tebuconazole on the AHR target genes *CYP1A1* and *CYP1A2* were analyzed in HepaRG cells by means of real-time RT-PCR analyses. Equimolar mixtures of both compounds were applied. Propiconazole generally appeared to be a slightly more potent activator of *CYP1A1* and *CYP1A2* transcription than tebuconazole (Fig. 5A and B). The mixtures of both fungicides induced the two CYP mRNAs, as well as CYP1A2 enzymatic activity in a concentration-dependent manner with high statistical significance (Fig. 5A–C). Modeling of the concentration-response curves of *CYP1A1* and *CYP1A2* mRNA induction demonstrated that the data were in line with the assumption of dose addition (Fig. 6).

4. Discussion

The present data demonstrate that tebuconazole is capable of activating AHR-dependent genes not only in rodent, but also in human liver cells. Data further indicate that tebuconazole is a weak activator of

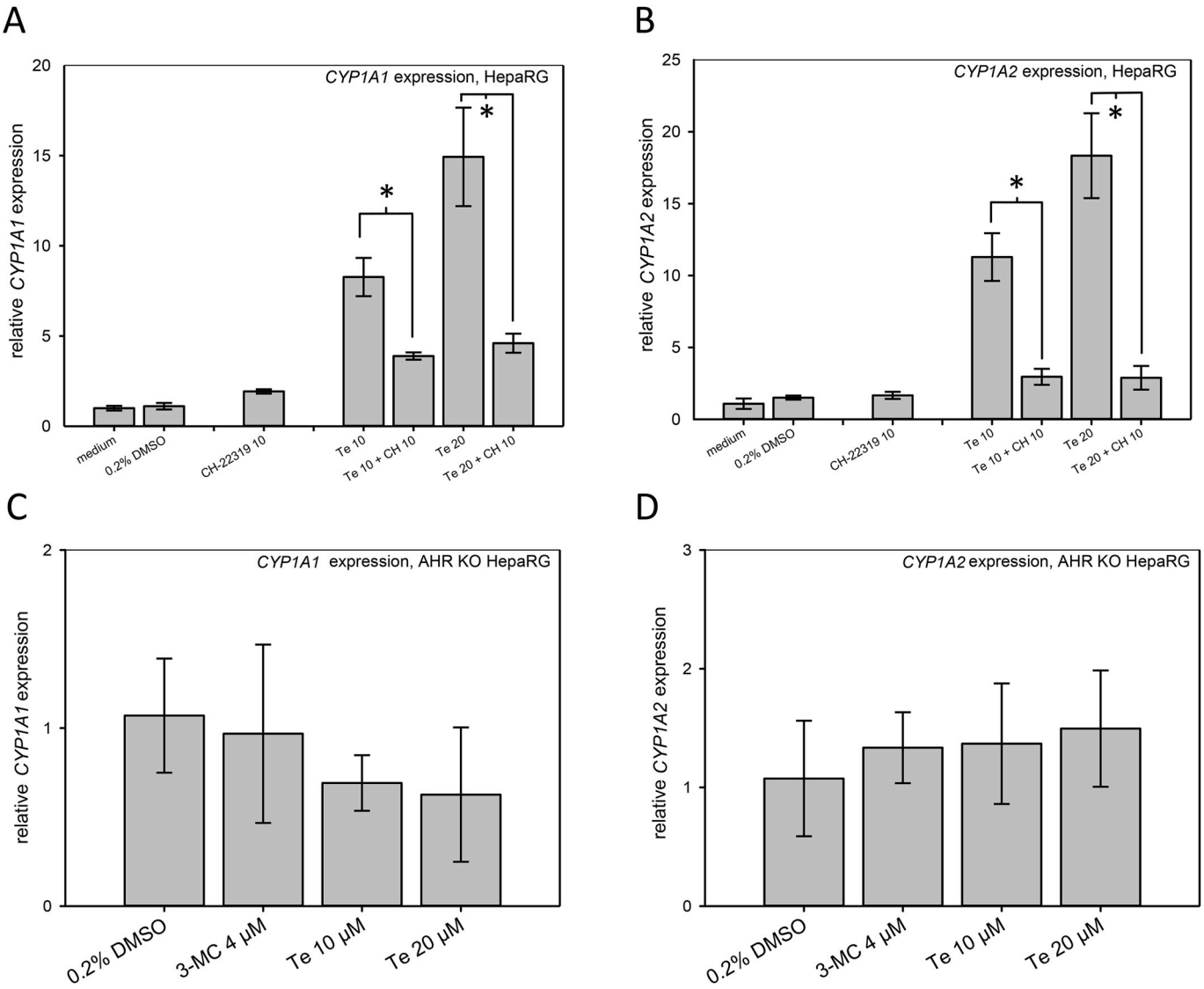

Fig. 3. The induction of *CYP1A1* and *CYP1A2* in HepaRG cells by tebuconazole (Te) is dependent on the AHR. Simultaneous treatment of HepaRG cells with tebuconazole (10 or 20 µM) and the AHR inhibitor CH-223191 (CH; 10 µM) significantly diminishes tebuconazole-induced upregulation of *CYP1A1* (A) and *CYP1A2* (B) mRNA levels. No induction of *CYP1A1* (C) and *CYP1A2* (D) expression by tebuconazole is observed in an AHR-deficient HepaRG variant. Mean ± SD (n = 3 independent experiments, each in three replicates) are depicted. Statistical significance ($p < 0.05$) is indicated by asterisks.

these genes, as compared to model AHR ligands and also in comparison with the structurally related fungicide propiconazole (Knebel et al., 2018a). The low micromolar concentrations at which first effects of tebuconazole on AHR-dependent genes were observed *in vitro* are similar to the tebuconazole concentration of approximately 5.8 µM in rat liver tissue after feeding of 1000 ppm tebuconazole, where comparable effects on AHR target genes were observed. In the course of the latter *in vivo* study, a slight increase in relative liver weight and centrilobular hypertrophy but no clear-cut adverse histopathological findings have been recorded (Schmidt et al., 2016). This indicates an onset of molecular effects on AHR target genes already at sub-toxic concentrations, both *in vivo* and *in vitro*. Nonetheless, the present data should not be used to conclude on AHR activation as the main causative mechanism of tebuconazole hepatotoxicity, because effects mediated by different molecular targets might play a role here.

Possible AHR activation by tebuconazole has not been addressed in detail before. The only reports available which potentially link the fungicide to AHR activation are a study by Sergent et al. (2009), which mainly focused on the effects of the pesticidal active compound imazalil on CYP1A1 induction, a study by Rieke et al. (2014) which has focused on the effects of various fungicides on hormone production but also included some aspects of AHR activation, and a study by Yang et al. (2018) which describes the activation of Cyp1a1/2 after treatment with tebuconazole in rats. As one of several comparators tebuconazole was used in the paper by Sergent et al. (2009) and was shown to induce CYP1A1 enzyme activity in intestinal Caco-2 cells. Effects of tebuconazole on other AHR-related endpoints, especially on the transcription of AHR target genes, however, were not analyzed. In the second study, *CYP1A1* induction has been studied in a placental cell line showing moderate induction of mRNA levels (Rieke et al., 2014) and in the third study (Yang et al., 2018), induction of protein levels and enzyme activity of Cyp1a1/2 were analyzed in rats. We now for the first time describe the activation of AHR-dependent genes in detail in human liver cells and also present data on effects in rats *in vivo*. The present results are in line with the abovementioned previous reports and substantially extend our knowledge about the molecular effects of tebuconazole and azole fungicide mixtures in human cells. With respect to the possibility of adverse effects in humans, the data presented here should, however, not directly be transferred to the situation of consumers, who are exposed to levels of pesticide residues via the diet which are expected to

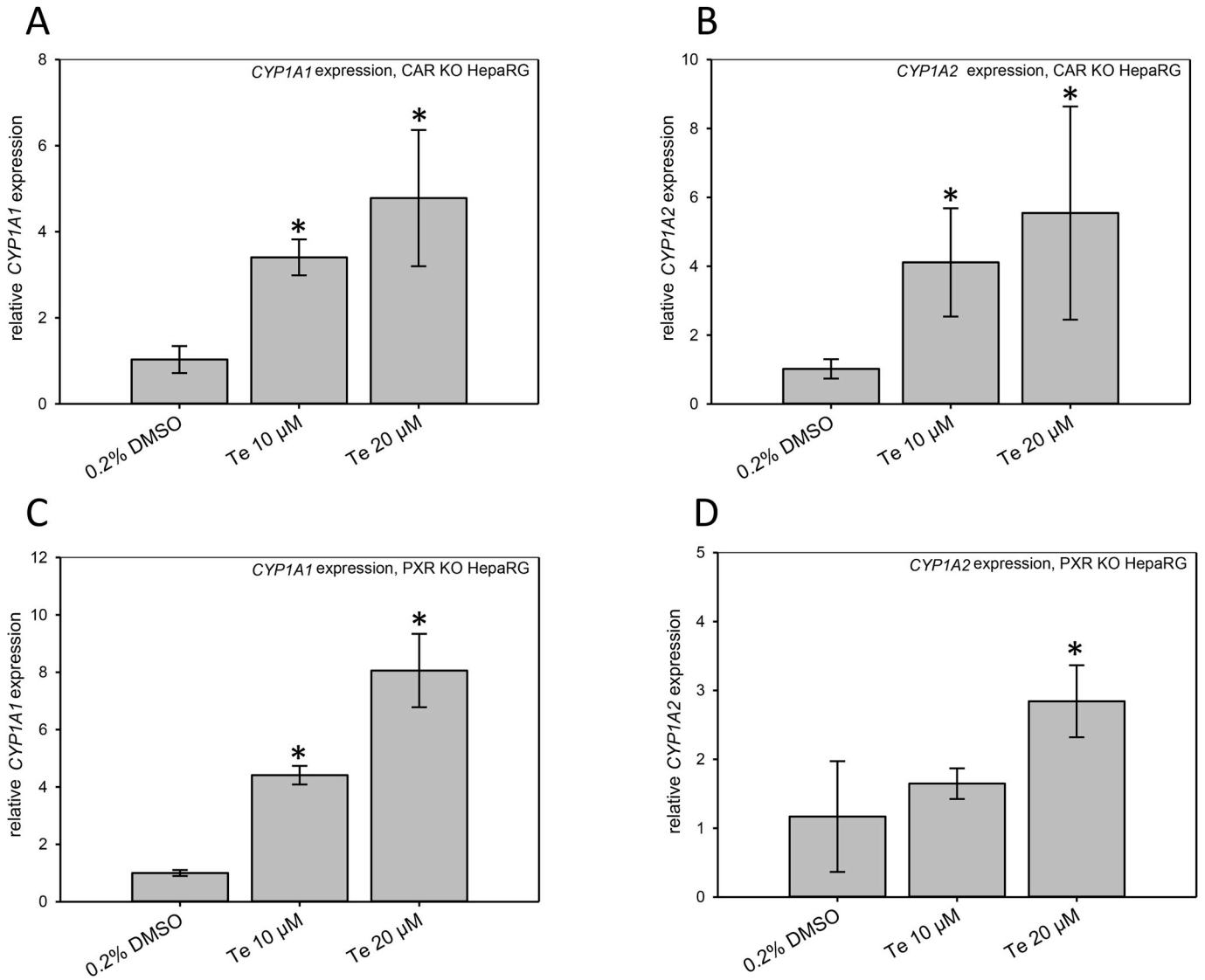

Fig. 4. Tebuconazole (Te) induces the expression of the AHR target genes *CYP1A1* and *CYP1A2* independent of CAR and PXR. Concentration-dependent induction of *CYP1A1* (A, C) and *CPY1A2* (B, D) is observed in HepaRG cells deficient in either CAR (A, B) or PXR (C, D). Mean ± SD (n = 3 independent experiments, each in three replicates) are depicted. Statistical significance ($p < 0.05$) is indicated by asterisks.

result in cellular concentrations several orders of magnitude lower than applied *in vitro* in the course of the study.

The observation of *CYP1A1* and *CYP1A2* induction by tebuconazole in the absence of a clear-cut response in AHR-dependent reporter gene assays warrants further discussion. Several scenarios might explain these seemingly discrepant findings. First, the sensitivity and dynamic range of the reporter gene assays used to detect AHR activation is less than for *CYP1A1* or *CYP1A2* mRNA induction. Thus the degree of AHR activation by tebuconazole in HepG2 cells which were the generally less responsive than HepaRG might have been too weak to provoke significant changes in reporter activities. Propiconazole turned out to induce *CYP1A1* mRNA induction in HepaRG approximately three times more potent than tebuconazole, whereas the difference was bigger (approx. 10-fold stronger induction by propiconazole) in HepG2 cells. As previously reported propiconazole stimulated the most sensitive AHR-dependent reporter system in HepG2 cells by approximately 17-fold (Knebel et al., 2018a). No effect on the same reporter system was detected here when the reporter assays was performed under identical conditions with tebuconazole. Given the aforementioned pronounced effect obtained with propiconazole (Knebel et al., 2018a), one might have expected a definitely weaker but still measurable induction of the reporter signal also for tebuconazole. This argues in favor of the hypothesis that insufficient sensitivity of the luciferase reporter assays was not causative for the lack of signal induction by tebuconazole. Instead, the compound may possibly not directly activate the AHR. Of note, and consistent with our present findings, Rieke et al. (2014) also observed *CYP1A1* mRNA induction but not AHR-dependent reporter gene activation by tebuconazole, epoxiconazole and flusilazole in placental cells. A second plausible scenario is that a tebuconazole metabolite may be responsible for AHR activation and not the parent compound. Our analyses with tebuconazole in combination with an external metabolizing system, however, did not reveal evidence for the latter scenario. Tebuconazole is rapidly metabolized in liver cells and this involves various hydroxylation steps followed by conjugation reactions (EFSA, 2014). Both types of reactions are expected to increase hydrophilicity and therefore rather to decrease the compound's interaction with the AHR which generally prefers planar and hydrophobic ligands (Abel and Haarmann-Stemmann, 2010). Even though the S9-mix external metabolic system might not fully reflect all relevant aspects of cellular metabolism and is additionally limited with regard to incubation times due to cytotoxic effects on HepG2 cells, the present data do not provide evidence for a major role of a tebuconazole metabolite in the activation

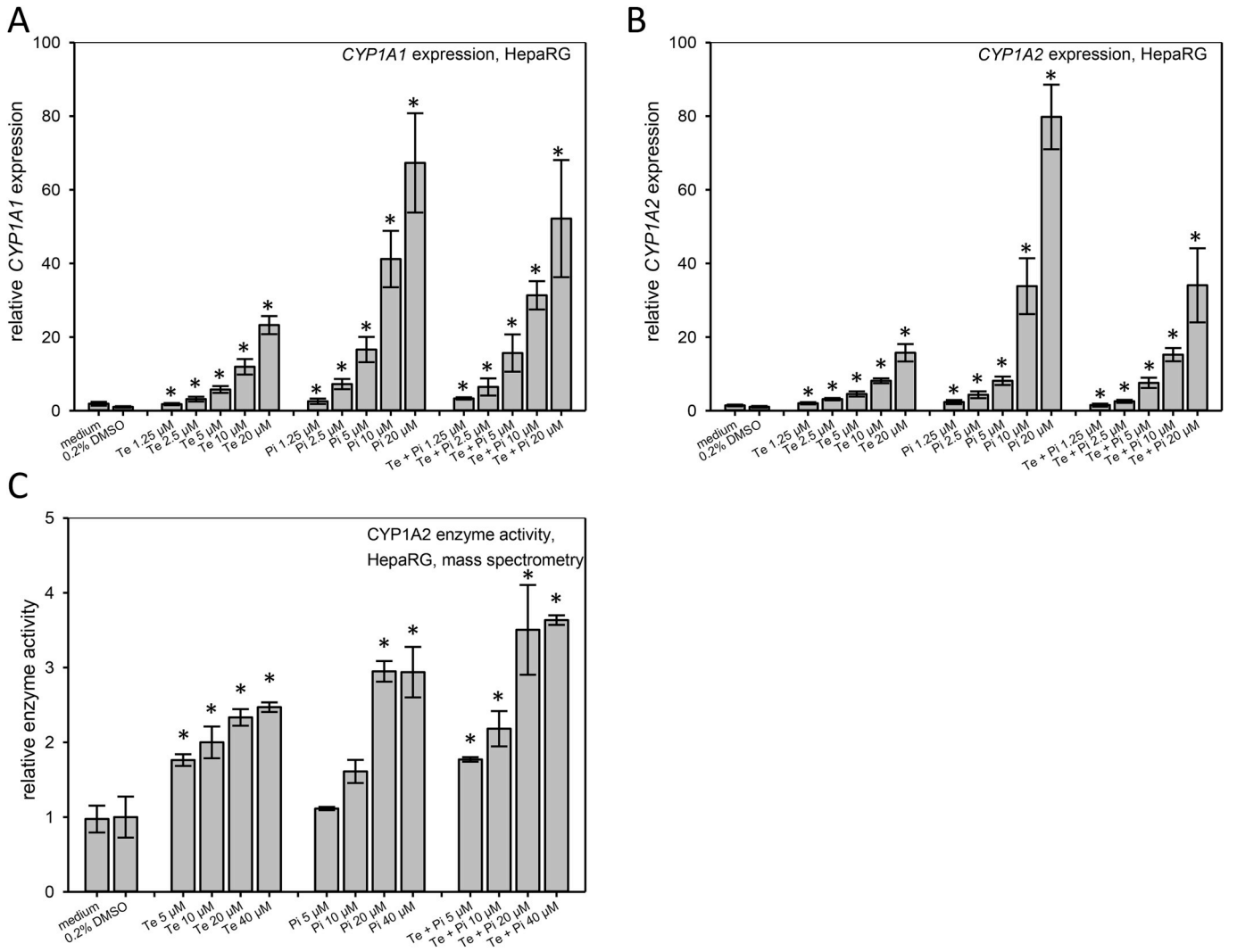

Fig. 5. Tebuconazole (Te) and propiconazole (Pi) exert additive effects on AHR target gene expression. *CYP1A1* (A) and *CYP1A2* (B) mRNAs, as well as CYP1A2 enzyme activities (C) are induced by propiconazole, tebuconazole, and their equimolar mixtures (please note that the concentrations given for the mixtures represent the sum of both pesticides; e.g. "Te + Pi 20 µM" means 10 µM Te + 10 µM Pi). Mean ± SD (n = 3 independent experiments, each in three replicates) are depicted. Statistical significance ($p < 0.05$) is indicated by asterisks.

of AHR-dependent transcription. It thus appears not unlikely that tebuconazole influences AHR-dependent genes by a mechanism different from direct AHR activation as a ligand. Imazalil, an imidazole-class fungicide, has been reported to induce CYP1A1 activity in human cells without involving AHR binding and without affecting CYP1A1 transcription (Sergent et al., 2009). Taken together these findings suggest that for the different fungicides, despite their chemical similarity and despite the fact that the compounds share a number of common target genes, the exact molecular mechanisms which contribute to alterations of a certain endpoint (i.e., altered CYP activity) might be different.

In addition to acting on target genes of one xenobiotic-sensing receptor by different ways, the members of the azole class of fungicides do not appear to be very specific activators of individual nuclear receptors: tebuconazole is an activator of PXR, but an antagonist of CAR activation (Knebel et al., 2018b); cyproconazole activates human PXR and RARα, while only rodent but not human CAR is activated by this compound (Luckert et al., 2018; Marx-Stoelting et al., 2017); propiconazole is an activator of CAR and PXR (Knebel et al., 2018b) as well as of the AHR (Knebel et al., 2018a); imazalil, as mentioned above, appears to have an indirect effect on the AHR target gene CYP1A1 without binding to the receptor (Sergent et al., 2009); and prochloraz, an imidazole-class fungicide, is thought to act via the AHR but possibly also via CAR (Halwachs et al., 2013; Marx-Stoelting et al., 2017; Rieke et al., 2014). Thus, the biological effects exerted by azole fungicides are suspected to be the consequence of a plethora of different individual target molecules such as, for example, the nuclear receptors. The situation gets even more complex when mixtures of pesticidal active compounds are examined, of which each single one is capable of influencing more than one single cellular target. Previous analyses of the effect of multiple pesticides on the AHR are sparse. Ghisari et al. (2015) have analyzed the effect of a mix of four compounds (terbuthylazine, propiconazole, cypermethrin, and malathion) *in vitro* and concluded on an additive behavior. Propiconazole was also used *in vitro* in mixtures with the prototypical AHR agonist benzo[*b*]fluoranthene, where also dose addition was observed (Knebel et al., 2018a). Similarly, *in vivo* data suggest that the induction of *Cyp1a1* in rat livers by combinations of cyproconazole, epoxiconazole, and prochloraz can be explained by dose addition (Heise et al., 2018). In placental cells combinations of tebuconazole, epoxiconazole and flusilazole also showed additive behavior with respect to *CYP1A1* mRNA induction (Rieke et al., 2014). The above findings are generally in line with the present observations which suggest dose addition of propiconazole and tebuconazole. Available data for AHR activation by pesticide mixtures thus suggest that the assumption of dose addition is sufficiently conservative to not

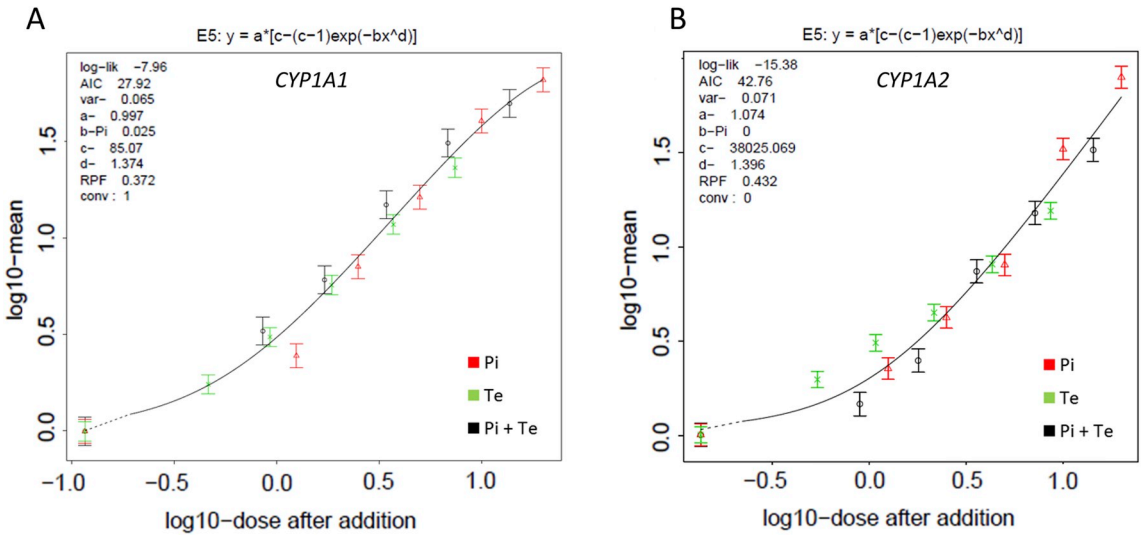

Fig. 6. Concentration-response modeling of the effects of tebuconazole, propiconazole and their equimolar mixtures on *CYP1A1* (A) and *CYP1A2* (B) mRNA levels. Modeling was performed using the software PROAST and the data presented in Fig. 5. Effects of the equimolar mixtures are in line with the assumption of dose additivity.

underestimate possible combination effects of azole-class pesticides at the AHR in human hepatic cells.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2018.11.039.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2018.11.039.

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