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***In vitro* differential responses of rat and human aryl hydrocarbon receptor to two distinct ligands and to different polyphenols**

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Abstract

TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) and several other environment/food-borne toxic compounds induce their toxicity via the aryl hydrocarbon receptor (AhR). AhR is also modulated by various endogenous ligands e.g. highly potent tryptophan (Trp)-derivative FICZ (6-formylindolo[3,2-b]carbazole) and natural ligands abundant in the human diet e.g. polyphenols. Therefore, evaluating AhR species-specific responses is crucial for understanding AhR physiological functions, establishing risk assessments, and exploring the applicability of AhR mediators in drug and food industry towards human-based usages. We studied AhR transactivation of FICZ/TCDD *in vitro* in a time-dependent and species-specific manner using dioxin responsive luciferase reporter gene assays derived from rat (DR-H4IIE) and human (DR-HepG2) hepatoma cells. We observed for the first time that FICZ potency was similar in both cell lines and was 40 times higher than TCDD in DR-HepG2 cells. Depleting

Trp-derivative endogenously produced ligands by using culture medium without Trp, resulted in 3-fold higher AhR activation upon adding FICZ in DR-H4IIE cells, in contrast to DR-HepG2 cells which revealed a fast degradation of FICZ induction from 10h post-exposure to complete disappearance after 24h. Seven polyphenols and a mixture thereof, chosen based on commercially recommended doses and adjusted to human realistic exposure, caused rat and human species-specific AhR responses. Two isoflavones (daidzein and genistein) induced rat AhR synergistic effects with FICZ and/or TCDD, while quercetin, chrysin, curcumin, resveratrol, and the mixture exerted a strong inhibitory effect on the human AhR. Strikingly, resveratrol and quercetin at their realistic nanomolar concentrations acted additively in the mixture to abolish human AhR activation induced by various TCDD concentrations. Taken together, these results illustrate the species-specific complexity of AhR transcriptional activities modulated by various ligands and highlight the need for studies of human-based approaches.

Keywords: Aryl hydrocarbon Receptor; Rat versus Human; Polyphenols; H4IIE cells; HepG2 cells

Capsule: Diet-borne endogenous and exogenous AhR modulators act differentially in human and rat hepatoma cells with quercetin and resveratrol being strong human AhR antagonists.

1. Introduction

The aryl hydrocarbon receptor (AhR) is known for its role as a xenobiotic mediator (Hankinson, 1995). Environment/food-borne contaminants like 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induce their toxicity via the AhR signaling pathway (Kawajiri and Fujii-KuriYama, 2016). Ligand (TCDD)-activated AhR undergoes a translocation from the cytosol to the nucleus to partner with the aryl hydrocarbon receptor nuclear translocator (ARNT). This AhR/ARNT heterodimer then binds to DNA sequences called dioxin responsive elements (DREs) to activate the expression of AhR inducible genes, including the cytochrome p450 (CYP) family (e.g. *CYP1A1*) belonging to the phase I drug-metabolizing enzymes. In addition to the AhR canonical pathway as described above, the AhR noncanonical pathway regulates the expression of genes lacking DREs by binding as a heterodimer with novel partners e.g. RelB or Krüppel-like Factor 6 via a site distinct from the consensus DREs, or importantly, via cross-talks with other signaling pathways (Wright et al., 2017). Nevertheless, the methods measuring DRE-regulated gene expression are widely accepted for determining AhR activation (Wall et al., 2015), among which cell-based screening methods such as luciferase reporter gene assays are amongst the most common (Scippo et al., 2004).

AhR is also activated by a wide range of endogenous and natural ligands to regulate several vital physiological functions in development and homeostasis, including immune responses, cell differentiation and proliferation, reproduction, and tumor suppression (Fujii-Kuriyama and Kawajiri, 2010). Indeed, AhR is considered as a sensor, connecting external environmental signals to cellular processes (Fujii-Kuriyama and Kawajiri, 2010; Marlowe and Puga, 2005). AhR functions can be dysregulated by inappropriate ligand-induced activation or inhibition, leading to subsequent health concerns (Marlowe and Puga, 2005; Xue et al., 2017).

Catabolism of the essential amino acid tryptophan (Trp) either by the host or by the symbiotic microbiota yields numerous physiologically relevant AhR ligands (Hubbard et al., 2015; Stejskalova et al., 2011). Among them, 6-formylindolo[3,2-b]carbazole (FICZ) is considered

the highest affinity AhR ligand to date with an estimated dissociation constant K_d of 0.07 nM, which is significantly lower than the TCDD K_d of 0.48 nM, measured in a competitive binding assay performed in rat hepatic cytosol (Rannug et al., 1987). FICZ is produced by photochemical reactions under ultraviolet and visible light (Fritsche et al., 2007; Wincent et al., 2009) or by enzymatic and oxidative pathways (Smirnova et al., 2016; Wincent et al., 2009), likely resulting in a systemic distribution of FICZ (Smirnova et al., 2016) to perform several essential functions especially in immunity (Rannug and Rannug, 2018). FICZ is able to activate AhR in various species such as fish (Jönsson et al., 2009), frog (Laub et al., 2010), and birds (Farmahin et al., 2014; Kim et al., 2016). However, little is known about its potency and mode of action compared to the widely studied AhR ligand TCDD.

On the other hand, polyphenols represent the largest source of AhR natural modulators in the human diet (Xue et al., 2017). They are secondary metabolites of plants with a common polyphenol structure and diverse chemical entities (Quideau et al., 2011). They are popular in research and food manufacture due to their significant antioxidant ability which could be useful to prevent various diseases associated with oxidative stress e.g. cancer, cardiovascular, and neurodegenerative diseases (Hardman, 2014; Karunaweera et al., 2015). Besides, they are also able to modulate the activity of several enzymes and receptors (Middleton et al., 2000). For example, polyphenols have been shown to affect AhR activities in several ways including directly as AhR functional agonists or antagonists, indirectly by interfering with AhR translocation, AhR-ARNT heterodimerization, AhR-DNA binding, or altering AhR co-activators and co-repressors (Xue et al., 2017).

While most risk assessment data for humans are based on rodent models, there are large interspecies differences in susceptibility to toxic effects induced by dioxins (Moriguchi et al., 2003), and potentially by these endogenous and natural AhR ligands. Therefore, evaluating AhR responses depending on species exposed is crucial to understanding the physiological

functions of this receptor, to establish risk assessment, and to apply AhR endogenous and natural mediators in drug and food industry towards human-based usages.

In this study, luciferase reporter gene cell-based assays involving rat and human hepatoma cells were applied to study the effect of FICZ versus TCDD and commonly commercialized polyphenols on AhR species-specific responses. Rat H4IIE and human HepG2 cells were used as species representatives since they contain the respective species-specific AhR along with the direct regulators of the AhR signaling pathways such as ARNT or co-factors, regardless of their cancerous cellular machinery. Moreover, while TCDD-elicited gene expression in rat H4IIE cells is consistent with *in vivo* rat studies (Dere et al., 2011), human HepG2 cells are considered amongst the best single-cell models described for predicting human toxicity (O'Brien, 2014), especially involving CYP1A1/2 activities (Choi et al., 2004). The aims of the present study were to: (a) investigate AhR species-specific (rat versus human) responses to FICZ and TCDD in the presence or absence of endogenous ligands by comparing medium with Trp and without Trp-the major source of AhR endogenous ligands, (b) evaluate effects of polyphenols and a mixture thereof, either alone or co-exposed with FICZ or TCDD to mimic realistic exposure to mixtures of AhR modulators, and (c) reveal the most active polyphenol(s) and possible interactions among these compounds in the mixture.

2. Materials and methods

2.1. Chemicals and suppliers

FICZ was bought from Sigma Aldrich (Missouri, USA), while TCDD was obtained from Wellington Laboratories (Ontario, Canada). Seven polyphenols: daidzein, quercetin, resveratrol, genistein, baicalin, curcumin, and chrysin were selected based on their commonly commercial availability as food supplements. They were purchased from Sigma Aldrich and dissolved in dimethylsulfoxide (DMSO) (Acros Organics, Molinons, France). Their detailed description is given in Table S1. A mixture of these seven polyphenols was designed to reflect

the realistic mixture exposure scenario of humans based on food supplement consumption or via their diet. The mixture contained each polyphenol at a concentration corresponding to a theoretical blood level arbitrarily calculated considering that the recommended ingested dose is diluted in five liters of blood, with an additional 1000-fold dilution factor to consider the intestinal absorption and the high metabolic rate of polyphenols. The nanomolar concentrations of the polyphenols in the mixture at blood level (Table S1) were relevant to human exposure (Williamson, 2017). The stock solutions and working solutions of the polyphenols and the mixture were stored at -20°C and protected from light. Six successive 2-fold dilutions were prepared freshly from the stock solution to obtain the tested concentrations which did not exceed 50 µM for the polyphenols and 65 µM for the mixture.

2.2. Determination of aryl hydrocarbon receptor transcriptional activities

2.2.1. Cell lines and luciferase reporter gene cell-based assays

Two dioxin responsive (DR) hepatoma cell lines were used, rat DR-H4IIE from BioDetection System (Amsterdam, The Netherlands) (DR-CALUX®) and human DR-HepG2 previously produced within the laboratory of Prof. Scippo (Liege, Belgium) (Van der Heiden et al., 2009). These cells were stably transformed with a vector containing four DREs, either from the mouse *Cyp1a1* promotor for DR-H4IIE cells or synthetic for DR-HepG2 cells, upstream of, respectively the mouse mammary tumor virus promotor (Garrison et al., 1996) or the thymidine kinase promotor (Van der Heiden et al., 2009) which control the transcription of a luciferase reporter gene. The cells were cultivated in MEM α (Thermo Fisher Scientific, Massachusetts, USA) with 10% v/v fetal bovine serum (Greiner, Kremsmünster, Austria) and 50 IU/mL penicillin and 50 µg/mL streptomycin (Sigma Aldrich), and were maintained in a water saturated atmosphere containing 5% CO₂ at 37°C.

We used the reporter gene assays to study the AhR transcriptional activity of FICZ compared to TCDD and of the polyphenols and the mixture alone or co-exposing with TCDD/FICZ EC₅₀

(more details about each experiment see section 3). The assays were carried out by following the method described elsewhere (Scippo et al., 2004; Van der Heiden et al., 2009). Briefly, after 24h of seeding in white clear-bottomed 96 well microplates (Greiner) with about 90% confluence in the well, the cells were exposed to the tested compound/mixture for various times in medium with Trp (MEM α , 10mg/l L-Tryptophan) or without Trp (DMEM w/o Trp, Biomol, Hamburg, Germany). Exposure was carried out with protection against direct light. The culture medium was replaced by freshly prepared medium with the tested compound/mixture. After exposure, the cells were washed with phosphate buffered saline (Sigma Aldrich) and lysed by a solution containing Triton X100 (Sigma Aldrich). Light production was triggered by adding luciferin (Promega, Wisconsin, USA) and ATP (Roche Diagnostics, Rotkreuz, Switzerland) to the cell lysate and measured by a luminometer (ORION II, Berthold Detection System, Pforzheim, Germany). Each experiment was repeated at least three times independently in triplicate wells, together with a reference curve of TCDD/FICZ for quality control. The final DMSO concentrations were 0.1% and 0.2%, respectively for the activation and the co-exposure experiments. Cytotoxicity was assessed by visual inspection of cell morphology, attachment and density under microscope.

2.2.2. Calculations of fold induction (FI), relative response (RR), EC₅₀, and IC₅₀

Luciferase activities in the reporter gene assays were recorded as relative light units (RLU). The final results in the activation tests were presented as fold induction (FI). In the co-exposure tests, the results were presented as relative response (RR). The FICZ/TCDD concentrations for co-exposures were chosen at their respective EC₅₀ to allow detection of further increase or decrease of the AhR activation induced by the half-maximum effect of TCDD/FICZ with the main focus on the inhibitory potency of the compound/mixture.

$$(1) FI_{i(activation)} = \frac{RLU_i}{RLU_{DMSO}}$$

$$(2) RR_{i(co-exposure)} = \frac{RLU_i - RLU_{DMSO}}{RLU_{TCDD/FICZ} - RLU_{DMSO}} \times 100$$

FI_i in an activation test was the ratio (fold) between the response of the tested compound/mixture (i) (RLU_i) and the response of DMSO in the same plate (RLU_{DMSO}) (Eq. (1)). Meanwhile, RR_i in a co-exposure test was the percentage (%) of the response of the cells to the tested compound/mixture (i) compared to the spike-in TCDD/FICZ EC_{50} , subtracting the baseline DMSO response (Eq. (2)). The non-linear regressions of four-parameter equations for agonistic activation (FI_i) (Eq. (3)) or antagonistic inhibition (RR_i) (Eq. (4)) were used to fit the dose-response curves (Graphpad PRISM software, version 7, San Diego, California, USA) where x was the concentration of the tested compound/mixture:

$$(3) FI_{i(activation)} = bottom + \frac{x^{hillslope}(top - bottom)}{x^{hillslope} + EC_{50}^{hillslope}}$$

$$(4) RR_{i(co-exposure)} = bottom + \frac{top - bottom}{1 + \frac{x^{hillslope}}{IC_{50}^{hillslope}}}$$

The corresponding EC_{50} and IC_{50} extrapolated by Graphpad PRISM from the full-dose response curves where available, were the concentrations of the tested compound/mixture exerting half their maximal effect (OECD, 2016). FI_{max} and RR_{max} were the maximum fold induction and the maximum relative response recorded within the tested concentration. Data from tests revealing cytotoxicity were excluded from the analyses.

3. Results

3.1. Aryl hydrocarbon receptor transactivation by FICZ and TCDD

The first experiment investigated the dose and time-dependent AhR transactivation by FICZ and TCDD in rat DR-H4IIE and human DR-HepG2 cells, considering the role of endogenously produced AhR ligands by using the standard medium containing Trp (MEM α) and the medium without Trp (DMEM w/o Trp). In general, FICZ strongly activated AhR to a similar extent than TCDD in both cell lines, however with different response patterns (Figure 1). A complete time-course (2h to 24h) comparison is displayed in Figure S1, while FI_{max} and EC_{50} values are in Figure S2.

The response of DR-H4IIE cells to FICZ and TCDD was similar and influenced by the presence of Trp in the culture medium. In MEM α with Trp, AhR activation was low ($FI_{\max} \cong 5$ fold) after the cells' exposure to both TCDD and FICZ (Figure 1, A1 and B1) with a gradual increase reaching $FI_{\max} = 12 \pm 0.2$ and 9 ± 0.2 fold, respectively, after 24h (Figure 1, C1). In DMEM w/o Trp, the cells' response to both FICZ and TCDD was 3-fold higher than in MEM α , e.g. after 6h exposure, $FI_{\max}$ was about 15 fold (Figure 1, B1), although the dose-response curves of the FICZ-induced activation started to degrade after 8h with much steeper curves (Figure S1, D1 to G1).

In contrast, the AhR transactivation in DR-HepG2 cells mainly depended on the added agonist (FICZ or TCDD). TCDD-induced human AhR activation in both culture media increased over time and reached a maximum of 21-fold induction after 24h exposure (Figure S1, A2 to G2). Meanwhile, the cells' response to FICZ increased during the first 4h post-exposure (Figures 1, A2 and S1, A2 and B2), remained stable between 4h and 8h (Figures 1, B2 and S1, B2 to D2), then decreased and completely vanished after 24h (Figures 1, C2 and S1, E2 to G2). This quick decreasing response resulted in steeper curves and low $FI_{\max}$ after 10h exposure, indicating a fast metabolism of FICZ in DR-HepG2 cells. The cells were also dramatically more sensitive to FICZ than to TCDD. For example, in DMEM w/o Trp, FICZ elicited $FI_{\max} = 11$ fold after 6h exposure to 250 pM FICZ, while at this time 10000 pM of TCDD was required to induce the same effect (Figure 1, B2), showing a 40-fold higher potency of FICZ compared to TCDD. The corresponding FICZ EC_{50} was 11 ± 2.4 pM, ~40-fold lower than TCDD $EC_{50} = 403 \pm 29$ pM (Figure S2, B2).

3.2. Aryl hydrocarbon receptor transcriptional activities of polyphenols and their mixture

The seven individual polyphenols and their mixture were tested for AhR transcriptional activities in rat DR-H4IIE and human DR-HepG2 cells in four different culture media conditions

(DMEM w/o Trp, MEM α , and DMEM w/o Trp added either FICZ or TCDD EC₅₀). Two exposure times (4h and 8h) leading to relatively stable AhR responses to FICZ and TCDD (section 3.1) were chosen for this experiment in order to avoid the complication of FICZ degradation through the metabolic feedback loop. The spike-in TCDD EC₅₀ for 4h and 8h exposure were, respectively 85 pM and 15 pM in DR-H4IIE cells, and 650 pM and 250 pM in DR-HepG2 cells, while for FICZ, EC₅₀ were, respectively, 10 pM and 30 pM for both cell lines.

The polyphenols and their mixture induced no cytotoxicity in both cell lines, except curcumin at 50 μ M in DR-H4IIE cells, which was excluded from the data presented. In general, their activity increased from 4h to 8h post-exposure and vanished after 24h (only tested in MEM α with Trp) in both cell lines, except chrysin which retained a moderate AhR activation in DR-HepG2 cells, after 24h exposure with FI_{max} = 3.4 $\pm$ 0.5 fold (data not shown). The dose-response curves induced by the seven polyphenols and their mixture in the rat DR-H4IIE and human DR-HepG2 cells are shown in Figures S3 and S4, respectively.

In rat DR-H4IIE cells, in DMEM w/o Trp, the two isoflavones daidzein and genistein were shown to be AhR agonists. Daidzein exerted a FI_{max} of 4.7 $\pm$ 0.4-fold, while the maximal fold induction for TCDD was 12-fold (Figure 2A). In the co-exposure experiment, daidzein surprisingly boosted the cell's responses to both FICZ and TCDD EC₅₀, remarkably increasing the response to FICZ EC₅₀ up to RR_{max} = 744 $\pm$ 63% and that to TCDD EC₅₀ up to RR_{max} = 361 $\pm$ 60% (Figure S3, A2). This resulted in a final 18 $\pm$ 3 and 9 $\pm$ 2-fold induction, respectively for FICZ and TCDD cotreatment, after the spike-in EC₅₀ reference subtraction (Figure 2A), showing an almost 4 and 2-fold increase compared to its respective agonistic activity alone. Genistein stimulated the rat AhR activation in both media after 8h exposure, with FI_{max} = 5.9 $\pm$ 1 and 7.6 $\pm$ 0.4 fold, respectively, for medium without and with Trp (Figure 2B). Genistein also exerted an enhanced effect, but only when co-exposed to FICZ EC₅₀ (RR_{max} = 782 $\pm$

154%) (Figure S3, B2), which resulted in a final 24 ± 1 -fold induction, showing a 4-fold increase compared to its agonistic activity alone (Figure 2B).

This mutual enhancement resulting in a several fold increase suggests the presence of a synergistic effect between the isoflavones and FICZ/TCDD in DR-H4IIE cells. In contrast, isoflavones displayed no significant activities in DR-HepG2 cells (Figure S4, A and B). Noticeably, in DR-HepG2 cells, daidzein, but not genistein, antagonized TCDD EC₅₀ (RR_{max} = ~40%), but slightly increased the response to FICZ EC₅₀ (Figure S4, A2), indicating a selective AhR inhibitory effect of daidzein on TCDD induction.

For the other polyphenols, in the agonistic tests, the flavonol quercetin and both flavones (baicalin and chrysin) exerted strong human AhR agonistic effects in DR-HepG2 cells regardless of the presence of Trp in the culture medium with FI_{max} = 3.2 ± 0.2 , 3.7 ± 0.1 , and 5.2 ± 0.2 fold, respectively after 8h exposure in DMEM w/o Trp (Figure 3A). A similar response pattern was observed when MEM α was used as exposure medium (Figure S4, C1 to E1). In DR-H4IIE cells, these polyphenols induced only a weak AhR activation of ~2 fold, at their highest concentrations, but only in MEM α , (Figure S3, C1 to E1). In the same medium, resveratrol exerted relatively similar AhR agonistic responses in both cell lines (Figures S3, G1 and S4, G1), similarly to the polyphenol mixture (Figures S3, H1 and S4, H1). However, in DMEM w/o Trp after 8h exposure, they did not exert any agonistic effect in DR-HepG2 cells (Figure 3A).

In co-exposure tests, only quercetin inhibited rat AhR activation by TCDD EC₅₀ with RR_{max} = $33 \pm 8\%$ after 4h exposure (Figure S3, C2). Similar to the two isoflavones, resveratrol and the mixture seem to have a synergistic activity with FICZ EC₅₀ in DR-H4IIE cells after 8h exposure (Figure S3, G2 and H2). In DR-HepG2 cells, curcumin was an AhR inhibitor which antagonized

both TCDD and FICZ EC₅₀, and even the DMSO baseline of the human AhR response, regardless exposure time (Figures 3 and S4, F1 and F2).

However, quercetin was the strongest human AhR antagonist. Quercetin at 3 μM totally abolished the activity of 30 pM FICZ (RR_{max} = 5 ± 3%) in DR-HepG2 cells, while 15.5 μM of chrysin and 65 μM of the mixture were needed for the same effect (Figure 3B). Baicalin and resveratrol caused the same antagonistic effects but to a lesser extent (RR_{max} ≅ 50% at 50 μM) (Figure 3B). Both flavones did not affect the human AhR response to TCDD EC₅₀, while quercetin antagonized strongly TCDD at 4h and 8h post-exposure, in presence of respectively 650 pM and 250 pM TCDD, showing a V-shaped response curve (Figures 3C and S4, C2). The lowest tested concentration of quercetin (0.3 μM) already reduced by half TCDD EC₅₀-induced AhR response of DR-HepG2 cells after 8h exposure or completely after 4h, while 3 μM quercetin totally ablated the AhR activity induced by TCDD EC₅₀ at both exposure times (Figures 3C and S4, C2). The cells' response increased to around RR_{max} = 50% at higher concentrations (30 μM and above), probably reflecting the agonistic activity of quercetin at these concentrations. Similarly to quercetin, only 6.3 μM resveratrol strongly antagonized TCDD EC₅₀ to RR_{max} = 25 ± 7% after 8h exposure in DR-HepG2 cells, but without a distinct V-shaped dose response curve (Figures 3C and S4, G2). Also, the polyphenol mixture strongly antagonized human AhR activation by TCDD EC₅₀ with a dose-response curve similar to the one of quercetin (Figure 3C). These results are summarized in Table S2.

3.3. Human aryl hydrocarbon receptor inhibitory activity of resveratrol, quercetin and the polyphenol mixture

The TCDD inhibitory potentials in human DR-HepG2 cells of resveratrol, quercetin, and the polyphenol mixture at low concentrations were further tested in DMEM w/o Trp for 8h exposure. Figure 4A shows that both polyphenols and the mixture exhibited a strong inhibitory effect on the human AhR activated by 650 pM (≅ EC₇₀) TCDD. While 5 μM resveratrol inhibited

the AhR activity induced by 650 pM TCDD to $RR_{max} = \sim 20\%$, 2.5 μM quercetin or the mixture at blood concentration ($\cong 2.5 \mu\text{M}$) was able to totally abolish this activity. Their TCDD inhibitory effects started at concentrations as low as 0.2 to 0.3 μM (Figure 4A), and IC_{50} values were 1 ± 0.08 , 0.6 ± 0.03 , and $0.8 \pm 0.06 \mu\text{M}$, respectively, for resveratrol, quercetin, and the mixture. Co-exposing the cells to these polyphenols or the mixture at the same concentration (5 μM) with various concentrations of TCDD (0.15 to 20 nM) (Figure 4B) revealed that resveratrol was less efficient (a reduction of only 30% of TCDD 20 nM induction was observed) than the mixture and quercetin which blocked totally TCDD-induced AhR activation, even at the highest TCDD concentration (20 nM).

The inhibitory effect on human AhR activation induced by TCDD EC_{50} of the mixture at 8h was predicted using concentration addition (CA) and independent action (IA) models (Doan et al., 2019) using the information of the four AhR antagonists namely, daidzein, quercetin, curcumin, and resveratrol. The calculation not only allows the prediction of a realistic combined effect, but also sheds light on the interactions among the polyphenols in the mixture and their contributions for the mixture effect. Detailed calculations are presented in supplementary information "Calculations of mixture effect and effect unit". Both the CA and IA models predicted a dose-response curve which closely overlapped the experimentally measured curve (Figure 4C). The IA model predicted an $IC_{50} = 3.5 \mu\text{M}$ close to 2.5 μM of the CA model, compared to a measured 0.8 μM IC_{50} . Quercetin and resveratrol contributed for 56% and 39%, respectively, to the mixture effect (Figure 4D).

4. Discussion

4.1. Aryl hydrocarbon receptor responses to FICZ and TCDD

This study is the first to show a direct and cross-species (rat and human) comparison of AhR transactivation induced by endogenous FICZ and exogenous TCDD ligands in hepatoma cells. We found that FICZ potency was similar on both rat and human AhR. However, FICZ

was 40-fold more potent than TCDD in DR-HepG2 cells (Figure 1, S1 and S2). In a mouse knock-in model where the endogenous mouse *Ahr* gene was entirely replaced with the human *AHR* gene, a reduction of TCDD-induced *CYP1A* family genes expression was observed, although limited AHR protein accumulation might account for the attenuated responsiveness of the model to TCDD (Moriguchi et al., 2003). This is in agreement with our previous finding showing that the human AhR has a lower affinity for TCDD than the rat AhR (Doan et al., 2019). There is currently no data available for interspecies (rat and human) comparisons of AhR responses to FICZ. Meanwhile, in an intraspecies comparison, the low affinity murine isoform (AhR^d) which is the result of an Alanine to Valine change at residue 375, showed a 4-fold lower binding affinity for TCDD than the AhR^b high affinity variant (Poland et al., 1994). Nevertheless, both variants exhibited similar sensitivity to FICZ, and FICZ functioned similarly between the two mouse strains, exerting no differences in inducing T helper 17 immune cells (Duarte et al., 2013). Additionally, the binding of different ligands (e.g. FICZ versus TCDD) has been suggested to cause different AhR conformational changes, resulting in different downstream consequences, e.g. in modulating the recruitment of various co-activators and transcription factors (Denison et al., 2011). For example, a comparison of TCDD and FICZ in an *in vivo* mouse model for infection by the influenza virus revealed that they differentially affected various cell-specific AhR activators, most likely due to the different conformational changes that they induced (Wheeler et al., 2014).

In this study, depletion of Trp in the medium (DMEM w/o Trp) did not affect the AhR responses in DR-HepG2 cells, but led to an almost 3-fold higher induction by both TCDD and FICZ after 4h to 10h treatment in DR-H4IIE cells (Figures S1, B1 to E1 and 1, B1). A “pure” AhR antagonist, GNF315 (Smith et al., 2011) at 10 μ M inhibited half (reduced 64%) of the basal luciferase signal of DR-H4IIE cells in MEM α after 6h treatment, but it had no significant effect (reduced only 16%) on the basal response in DR-HepG2 cells (data not shown). Thus, it is likely that on top of the activities of the FICZ added, the rat AhR responded to the high level

of basal AhR activation caused by endogenously produced ligands derived from Trp upon addition of the fresh medium. These ligands were unstable, as the difference in medium with and without Trp tended to vanish after 12h treatment (Figure S1, F1 to G1).

In DR-HepG2 cells, AhR transactivation of both TCDD and FICZ was not affected by the presence of Trp in culture medium (Figure 1, A2 to C2). This could result from a lower conversion rate of Trp to AhR endogenous ligands, or conversion to different compounds. Moreover, we showed that FICZ clearance was high in this cell line. AhR ligands, except metabolically stable ligands, activate AhR to induce their own metabolism via the induction of AhR downstream regulated genes, and subsequently, their clearance from the body (Chiaro et al., 2007). FICZ, which is rapidly metabolized by CYP1 isoenzymes (Wincent et al., 2009), causes only transient AhR signaling, while TCDD induces persistent AhR responses. The different susceptibility to metabolic feedback loop likely determines the mode of action of TCDD and FICZ (Duarte et al., 2013). Therefore, it appears that if FICZ clearance is blocked, such as by inhibiting the FICZ/AhR/CYP1A1 feedback loop, FICZ could also induce toxicity similar to that of TCDD, which have been observed *in vivo* in zebrafish embryos (Wincent et al., 2016). Thus, the metabolic feedback loop functions to prevent the toxicity of FICZ and possibly other labile AhR agonists (Brown et al., 2015; Wincent et al., 2016).

It is at present difficult to pinpoint the reason for the ligand-intrinsic differences in AhR responses while different susceptibility to metabolism complicates the issue. However, the conservation of high FICZ potency in inducing AhR activation across different species and among different AhR isoforms suggests its physiological importance as an endogenous AhR ligand during evolution (Laub et al., 2010).

4.2. Aryl hydrocarbon receptor responses to polyphenols and their mixture

This study, for the first time, reports the species-specific AhR transcriptional activity in cells exposed to different polyphenols. Isoflavones (daidzein and genistein) were more active in the

rat DR-H4IIE cells, while the flavonol quercetin, flavones (baicalin and chrysin) and curcumin strongly affected the human AhR activity in DR-HepG2 cells. The stilbene resveratrol acted equally on both cell lines. Although further investigations involving larger numbers of polyphenols are necessary to evaluate the AhR species-responses of different polyphenol subclasses, the data are valuable for selecting polyphenols as active compounds specifically for human-aimed research and also raise a concern for traditional rodent-to-human extrapolation in risk assessments. It is important to stress here that these activities were only observed after short term exposure (4h or 8h), only chrysin maintained some of its activity after 24h.

The two isoflavones (daidzein and genistein) (Figure 2) exerted AhR agonistic effects in DR-H4IIE cells and dramatically enhanced the cells' response in the presence of FICZ EC₅₀ (4-fold higher than acting alone). Several previous studies revealed a stronger AhR induction due to the interference of polyphenols with the clearance of FICZ via the FICZ/AhR/CYP1A1 feedback loop (Choi and Kim, 2008; Froyen and Steinberg, 2016), however we chose to perform our experiments at 4h and 8h post-exposure, before FICZ degradation kicks in, therefore we most likely can rule out this mechanism for the observed synergistic effects. An enhanced induction was previously observed for the glucocorticoid receptor-mediated transcription due to sodium arsenite-induced chemical stress, or for progesterone or androgen stimulation by a combined treatment with forskolin (an activator of adenylate cyclase) and trichostatin A (an inhibitor of histone deacetylases) (Willemssen et al., 2005). Likewise, a rat AhR synergistic activity of genistein with 300 pM TCDD was observed previously that was explained by the various cellular functions of genistein (Van der Heiden et al., 2009). Thus, in our case, the synergistic effects of daidzein and genistein co-exposing with FICZ EC₅₀ were likely due to their activity on other pathway(s) which helped to enhance AhR transactivation more efficiently. Note that both daidzein and genistein are estrogenic compounds which are able to bind to the estrogen receptor (ER) (Tapiero et al., 2002), while cross-talk between AhR and ER has been well described (Matthews and Gustafsson, 2006; Safe and Wormke, 2003).

Thus, it is possible that the synergistic activity of genistein and daidzein on the rat AhR could result from the cross-talk between AhR and ER.

The flavonol quercetin and the two flavones (baicalin and chrysin) (Figure 3) exerted significant AhR agonistic and antagonistic effects in DR-HepG2 cells but not in DR-H4IIE cells. Several mechanisms could explain the AhR agonistic activity of quercetin. Quercetin was reported as an inducer of CYP1A1 in human CaCo-2 cells, and the authors also showed the interaction of quercetin with AhR using *in silico* tools (Jin et al., 2018). In addition, quercetin was shown to significantly increase the AhR translocation by activating protein kinases C in rats, facilitating the transcriptional activity of AhR in osteoblasts (Sharan et al., 2011). Phosphorylation by e.g. protein kinases C is required for AhR activity in DNA binding and gene transactivation and for the transformation of the unliganded AhR into a fully functionally active AhR/ARNT heterodimer (Puga et al., 2009).

AhR antagonism of quercetin and chrysin at concentrations higher than 5 μ M was previously shown for DR-HepG2 cells after 6h exposure (Van der Heiden et al., 2009). However, another original finding of this study is the strong AhR antagonistic activity of quercetin but at very low concentrations ($\leq 5 \mu$ M) in DR-HepG2 cells which were co-exposed to quercetin and increasing concentrations of TCDD (Figure 4, A and B). One could suggest that quercetin is a TCDD competitive antagonist, strongly competing to TCDD for binding sites on the human AhR, similarly to alpha-naphthoflavone, a well-known AhR antagonist (Blank et al., 1987). Quercetin was also found to induce AhRR (AhR repressor) mRNA expression in Caco-2 cells, thereby facilitating AhRR-dependent transrepression of AhR and inhibiting the BaP-AhR activation via AhRR (Niestroy et al., 2011). However, there could be additional underlying mechanism(s) for the strong antagonistic effect of quercetin in human DR-HepG2 cells, which deserves further investigations.

Resveratrol was an AhR agonist in both cell lines (Figures S3, G1 and S4, G1). It also enhanced the response to FICZ of the rat AhR in the same manner as the two isoflavones (Figure S3, G2). In addition, it was a strong AhR antagonist in DR-HepG2 cells, although less potent than quercetin (Figures 3, B and C and 4, A and B). Resveratrol was found to not exhibit AhR agonistic or antagonistic activity in human breast cancer T47D or MCF-7 cells (Lee and Safe, 2001). Instead, it inhibited *CYP1A1* mRNA expression and CYP1A1-dependent activity via an AhR-independent post-transcriptional pathway. It promoted the nuclear translocation and/or DNA-binding activity of AhR, showing partial AhR-agonistic features. Meanwhile, it reduced AhR-inducible gene expression without interfering with AhR-DRE binding, displaying its AhR-antagonistic effect when cells were co-exposed to TCDD. Also, CYP1A1 repression by resveratrol was shown to be dependent on ER since siRNA ER α was able to completely abolish the repressive effect of resveratrol on TCDD-inducible CYP1A1 mRNA accumulation in MCF-7 cells, but not in Caco-2 cells, indicating cell-specificity (Perdew et al., 2010).

We showed that curcumin (Figure 3) exerted an AhR antagonistic activity only in human DR-HepG2 cells. In mouse Hepa-1c1c7 cells, curcumin was reported to accelerate the TCDD-induced AhR translocation, but inhibit the heterodimerization of AhR with ARNT by dose-dependently inhibiting the TCDD-induced phosphorylation of both AhR and ARNT, thereby suppressing AhR activation (Nishiumi et al., 2007). Furthermore, oxidative stress was shown to be involved in mediating the curcumin-induced degradation of AhR and ARNT in human Hep3B and MCF-7 cells (Choi et al., 2008). These mechanisms might be less involved, or carried out by different cell components in the rat DR-H4IIE cells (Dere et al., 2011), thus explaining the nearly complete absence of AhR antagonism of curcumin in this study. To conclude, it seems that the effect of polyphenols on the AhR transcriptional activities is far more complicated since they rarely act as a direct AhR ligand only, but instead involve in several complex cellular pathways.

The mixture of the seven polyphenols exerted an AhR agonistic activity in both cell lines. Interestingly, similarly to quercetin, it also inhibited strongly the AhR activation induced by TCDD at various concentrations in DR-HepG2 cells (Figure 4B). Both CA and IA models resulted in an acceptable prediction of the antagonistic mixture effect (Figure 4C), indicating that the activities of these polyphenols were additive in the mixture with similar or dissimilar modes of action, in agreement with the conclusion of multiple intracellular targets of the polyphenols.

Quercetin and resveratrol acted together, contributing to 95% of the AhR antagonistic mixture effect, which was able to totally abolish the AhR activation of 650 pM TCDD in DR-HepG2 cells at a concentration of 2.5 μ M (Figure 4A). At this concentration, the mixture contained 0.33 μ M quercetin and 0.44 μ M resveratrol, corresponding to theoretical calculated blood levels (Table S1). According to literature, these concentrations could be realistic in human blood after dietary polyphenol ingestion. Quercetin is naturally found in onions, apples, broccoli, and berries (Li et al., 2016), while resveratrol is abundant in red grapes, red wine, peanuts, and ground nuts (Stervbo et al., 2007). Two main metabolites of quercetin, quercetin-30-sulphate and quercetin-3-glucuronide, accumulated in human plasma with maximum concentration of 0.665 μ M and 0.351 μ M respectively after less than an hour after the ingestion of 270 g fried onions (Mullen et al., 2006). These two metabolites were found to display antitumor effects in MCF-7 cells, comparably to quercetin (Wu et al., 2018). Similarly, the blood concentration of resveratrol can peak at 2 μ M after an hour of single administration of an oral dose of 25 mg of *trans*-resveratrol to healthy volunteers (Romero-Pérez et al., 1999).

In our previous study (Doan et al., 2019), we showed that a mixture of 29 persistent organic pollutants, the total POP mixture prevalent in Scandinavian human blood, also caused an AhR antagonistic effect, which could be realistic. While this total POP mixture can cause a persisting AhR antagonism, the AhR antagonistic effects of quercetin, resveratrol, and the

polyphenol mixture are only transient due to their fast metabolism. However, the health impact of the human exposure to these multiple chemicals responsible for AhR antagonism should be assessed.

Conclusion

We showed for the first time a side-by-side comparison of the effects of an endogenous AhR ligand (FICZ) and exogenous ligand (TCDD) on two hepatoma cell lines from two different organisms (rat and human). FICZ was 40 times more potent than TCDD in human DR-HepG2 cells and displayed similar potency in both cell lines, indicating its physiological importance as an endogenous AhR ligand. Furthermore, we showed a significant effect of Trp depletion on AhR activation in rat DR-H4IIE cells, but none in DR-HepG2 cells, probably due to the higher sensitivity of the former to the endogenously produced ligands from Trp, along with a faster degradation of the ligand FICZ in the latter. The effect of seven polyphenols and their mixture on AhR transcriptional activities alone and co-exposing with FICZ or TCDD was also investigated. Both agonistic and antagonistic effects were observed, in line with the expected complexity of a system where differential degradation of the tested compounds was certainly playing a role, as well as interactions of the compounds, and their metabolites, with various components of the cellular machinery. Antagonistic activity of quercetin, resveratrol, and the mixture towards TCDD-induced human AhR activation was observed in this study at nanomolar concentration, which is realistic in human exposure levels.

FICZ and other endogenous ligands together with polyphenols, in concert, regulate and maintain the vital physiological functions of AhR in balance. This balance can be disrupted by xenobiotic exposure or mis-applications of natural supplements but also can be used to treat diseases related to AhR disfunctions. Further studies are required to fully understand the interference of these dietary polyphenols on the AhR-mediated activity of both endogenous

and persistent xenobiotic AhR ligands in order to assess the health impact of their use as drugs or food supplements.

Conflict of interest: The authors declare that there is no conflict of interest.

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★ TCDD MEM α ● TCDD DMEM w/o Trp
 ★ FICZ MEM α ● FICZ DMEM w/o Trp

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750 Figure 1. Dose response curves of rat DR-H4IIE and human DR-HepG2 cells exposed to
 751 TCDD or FICZ in culture medium with (MEM α) and without Trp (DMEM w/o Trp) at 2h (A), 6h
 752 (B) and 24h (C) post-exposure (Mean $\pm$ SD, n = 3).

Figure 2. Dose response curves of rat DR-H4IIE cells responding to (A) daidzein or (B) genistein in medium with Trp ($\text{MEM}\alpha$), without Trp (DMEM w/o Trp) or cotreated with FICZ or TCDD EC_{50} in DMEM w/o Trp for 8h induction (Mean $\pm$ SD, $n = 3$). TCDD Max: maximum response of TCDD at the same condition.

Figure 3. Dose response curves of human DR-HepG2 cells responding to quercetin, baicalin, chrysin, curcumin, resveratrol, or the polyphenol mixture in (A) medium without Trp (DMEM w/o Trp) or cotreated with (B) FICZ EC_{50} or (C) TCDD EC_{50} in DMEM w/o Trp for 8h induction (Mean $\pm$ SD, n = 3). TCDD Max: maximum response of TCDD at the same condition.

Figure 4: Dose response curves of DR-HepG2 cells responding to (A) the mixture, quercetin, and resveratrol co-exposed with 650 pM TCDD and (B) various TCDD concentrations co-exposed with 5 μ M of these polyphenols. (C) The predicted and measured dose response curves of the mixture after 8h induction. (D) Distribution of effect units of the four active AhR antagonists in the mixture. CA: concentration addition. IA: independent action.