



In vitro toxicity and *in silico* docking analysis of two novel selective AH-receptor modulators[☆]



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ABSTRACT

The mediator of dioxin toxicity, aryl hydrocarbon receptor (AHR), has also important physiological functions. Selective AHR modulators (SAHRMs) share some effects of dioxins, except for their marked toxicity. We recently characterised toxicologically two novel SAHRMs, prodrugs IMA-08401 and IMA-07101 in rats, demonstrating that they are far less deleterious than the most toxic AHR-agonist, TCDD. Here, we analysed the *in vitro* toxicity and *in silico* AHR binding of the respective active, deacetylated metabolites, IMA-06201 (N-ethyl-N-phenyl-5-chloro-1,2-dihydro-4-hydroxy-1-methyl-2-oxo-quinoline-3-carboxamide) and IMA-06504 (N-(4-trifluoromethylphenyl)-1,2-dihydro-4-hydroxy-5-methoxy-1-methyl-2-oxo-quinoline-3-carboxamide). In H4IIE rat hepatoma cells, IMA-06201 and IMA-06504 induced CYP1A1 with comparable potencies and efficacies to those of TCDD. They had little effect on cell viability as assessed by LDH leakage and MTT reduction assays, and were not mutagenic in the Ames test, but IMA-06504 elicited a maximally 2.7-fold increase in micronuclei. Molecular docking simulations showed that similar to TCDD, they occupy the central region of AHR ligand binding cavity. Hence, while showing low to negligible *in vitro* toxicity, these novel SAHRMs bind to the AHR qualitatively in a similar fashion to TCDD, and appear comparably powerful AHR agonists. Combined with our earlier results demonstrating that they seem considerably less toxic *in vivo* than TCDD, these compounds are thus highly interesting new SAHRMs.

1. Introduction

The aryl hydrocarbon receptor (AHR) is an evolutionarily ancient, ligand-activated transcription factor (Beischlag, et al. 2008). It regulates the activity of various genes in different cell types across all vertebrates, including genes for some xenobiotic-metabolising enzymes, “the AHR battery”. The AHR has varied, important physiological functions, and is also well known and extensively studied as the mediator of toxicity induced by a class of environmental contaminants called dioxins [polychlorinated dibenzo-*para*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and dioxin-like polychlorinated biphenyls (PCBs); reviewed, for example, in (Hahn and Karchner 2011, Mandal 2005)].

The molecular mechanism of AHR action has been revealed in detail for transcriptional induction of the drug-metabolising enzyme CYP1A1,

which is believed to represent a more general pattern, known as the canonical pathway of AHR signalling. In its inactive state, the AHR is located in the cytosol in association with the chaperone proteins HSP90, XAP2 and p23. Binding of a ligand triggers transformation in the protein structure, causing the AHR to translocate into the nucleus. There it sheds the cytosolic protein partners and dimerizes with a structurally related protein, ARNT. The AHR-ARNT dimer then binds to the DNA at specific enhancer sites called dioxin response elements (DREs) in the promoter region of the *Cyp1a1* gene. This eventually leads to induced transcription of CYP1A1 mRNA (Ma 2011), a fairly rapid and highly sensitive marker for AHR activation (Abraham, et al. 1988). Although the canonical signalling pathway is in general quite well understood, many details, especially about its regulation, are still lacking. Furthermore, in addition to the canonical pathway, there appear to be several non-canonical pathways related to AHR function,

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which involve the AHR interacting with other transcription factors or pathways within cells (Denison and Faber 2017, Guyot, et al. 2013).

The AHR binds numerous ligands, which have notably diverse structures and can be of both exogenous and endogenous origin. This promiscuity is considered to be at least partly due to differences in binding modes to the ligand binding domain (LBD) of the AHR (DeGroot et al., 2011). It is particularly interesting because the effects of differential AHR modulation are highly ligand-specific (Denison and Faber 2017) and of great variety, ranging from beneficial to extremely toxic. Furthermore, while AHR homologues are widespread in fauna, their functions among species vary. There are genes, such as *Cyp1a1*, whose expression is modulated consistently by the AHR across species in response to AHR modulators; however, there are also various others, whose expression differs significantly among species, in some cases even among strains (Denison and Faber 2017, Flaveny, et al. 2010, Forgacs, et al. 2013, Pohjanvirta, et al. 1993, Sun, et al. 2004). The strain and species differences appear, for a large part, to be attributable to structural differences of the AHR (Denison, et al. 2011, Pohjanvirta, et al. 2011). However, those may not be adequate to alone explain the diverse outcomes of AHR modulation observed within organisms by various AHR agonists. It is likely that differences in mechanistic steps related to AHR ligand or DNA binding, interactions with transcriptional co-regulators, subsequent signalling pathways, and their regulation are also important (Bonati, et al. 2017, Gasiewicz and Henry 2011).

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the most toxic dioxin and has, as such, been widely employed in research as a classical compound for activation of the AHR (Van Den Berg, et al. 1998). TCDD causes a multitude of adverse effects in laboratory animals including hypophagia, wasting syndrome, developmental toxicity, endocrine disruption, carcinogenicity and immunotoxicity (Pohjanvirta and Tuomisto 1994). The current consensus is that they result from inappropriately persistent and untimely activation of the AHR (Bock and Köhle 2006, Denison, et al. 2011). Interestingly, some of the biological impacts of TCDD-induced AHR modulation are such that they would be beneficial in the treatment of certain conditions, if toxicity could be evaded (Vorderstrasse and Lawrence 2006, Xu, et al. 2015). Furthermore, increasing evidence is emerging about the involvement of the AHR in normal physiological functions and in disease aetiology, particularly in relation to immunomodulation and cancer (Feng, et al. 2013, Murray, et al. 2014, Quintana, et al. 2010, Zhu, et al. 2014).

So far, appropriate modulation of AHR activity has been shown as a potential target for novel therapeutics in the treatment of, for instance, cancer (Díaz-Díaz, et al. 2016, Jin, et al. 2014), multiple sclerosis (MS) (Singh, et al. 2007), inflammatory skin diseases (Di Meglio, et al. 2014, Haas, et al. 2016, Van Den Bogaard, et al. 2013), Crohn's disease (Benson and Shepherd 2011) and inflammatory bowel disease (Arsenescu, et al. 2011, Furumatsu, et al. 2011). Therefore, the AHR appears a highly interesting target for novel therapies in several fields, and selective AHR modulators (SAHRMs) intriguing candidates for lead compounds. SAHRMs are a large and diverse group of both natural and engineered molecules, which induce subsets of AHR-mediated effects, often without the major toxic outcomes of dioxins such as TCDD. In addition, SAHRMs could be valuable tools in further elucidating the multifaceted physiological roles of the AHR and the underlying molecular mechanisms.

We recently characterised the toxicity of two novel such SAHRMs in Sprague Dawley (SD) rats: IMA-08401 (*N*-acetyl-*N*-phenyl-4-acetoxy-5-chloro-1,2-dihydro-1-methyl-2-oxo-quinoline-3-carboxamide; later referred to as C2 for simplicity; Fig. 1) and IMA-07101 (*N*-acetyl-*N*-(4-trifluoromethylphenyl)-4-acetoxy-1,2-dihydro-5-methoxy-1-methyl-2-oxo-quinoline-3-carboxamide; later C4) (Mahiout, et al. 2017). C2 and C4 represent diacetyl prodrugs of *N*-hydrogen metabolites of the immunomodulatory drug compounds laquinimod and tasquinimod, of which laquinimod has been studied in phase II/III clinical trials for treatment of MS (Polman, et al. 2005, Thöne and Linker 2016) and Chron's disease (D'Haens, et al. 2015), and is currently in phase II

studies for treatment of Huntington's disease (García-Miralles, et al. 2016). *In vivo*, we demonstrated that even at the highest doses practically achievable, C2 and C4 appeared considerably less toxic than TCDD (at a some 1000-fold lower single dose) after acute and subacute (repeated 5-day) dosing, while acting as effective activators of the AHR, as evaluated by *Cyp1a1* gene induction (Mahiout, et al. 2017). Some major characteristic toxicities of dioxins that C2 and C4 lacked, at least at the dose levels tested, were hypercholesterolemia, reduced plasma thyroxine levels, acute lethality, wasting syndrome, grave liver and testis lesions, hypoglycaemia, and elevated plasma free fatty acid (FFA) levels (Pohjanvirta, et al. 2011, Viluksela, et al. 1999). The main adverse effects seen with high doses of C2 and C4 *in vivo* were thymic atrophy, alterations in serum triglyceride and 3-hydroxybutyrate levels, and changes in liver and kidney retinol and retinyl palmitate concentrations (Mahiout, et al. 2017). Furthermore, there were modulations in the expression of selected genes of the AHR battery. Intriguingly, all of the dioxin-like effects observed *in vivo* fell into the “type I” category previously demonstrated to be largely indifferent to structural variation at the transactivation domain of the AHR in TCDD-treated rats (Pohjanvirta, et al. 2011).

To gain further understanding of the effects and potency of these compounds, in the present study we characterised *in vitro* effects of the respective active, deacetylated metabolites that the prodrugs C2 and C4 readily hydrolyse to *in vivo*: IMA-06201 (*N*-ethyl-*N*-phenyl-5-chloro-1,2-dihydro-4-hydroxy-1-methyl-2-oxo-quinoline-3-carboxamide; later C1) and IMA-06504 (*N*-(4-trifluoromethylphenyl)-1,2-dihydro-4-hydroxy-5-methoxy-1-methyl-2-oxo-quinoline-3-carboxamide; later C3). C1 and C3 are, despite being the active compounds, unsuitable for *in vivo* formulations due to their low aqueous solubility, hence the use of the prodrugs C2 and C4 *in vivo* and C1 and C3 *in vitro*. The chemical relationships between C2, laquinimod and C1 are depicted in Fig. 2.

C1 and C3 were first assessed for their *in vitro* toxicity, in some cases in parallel with TCDD, to further compare their effects. Generally, TCDD is not overtly toxic to cultured cells *in vitro*, despite its drastic effects *in vivo* (Beatty, et al. 1975, Knutson and Poland 1980, Schecter, et al. 1987). However, the same could not be taken for granted for C1 and C3, particularly because *in vivo*, we had observed some effects with C2 and C4 that have not been reported with TCDD. Those included a conspicuous hyperaemia of the ears, minimal extramedullary myeloid haematopoiesis in the liver, a reduction of serum triglycerides, and an increase of serum 3-HB (Mahiout, et al. 2017).

Here, the endpoints included were cytotoxicity assessed by LDH leakage assay, reduction of cell viability/metabolic activity by MTT reduction assay, and genotoxicity by Ames and micronucleus tests. Furthermore, the AHR activation potency and efficacy of C1 and C3 were studied by measuring CYP1A1 enzyme activity in the metabolically active rat hepatoma cell line H4IIE, and compared with those of TCDD. We also examined the ability of a specific, ligand-selective AHR antagonist, CH-223191, to block CYP1A1 induction by these compounds. Finally, we explored and compared the abilities of C1, C3, and TCDD to bind the rat AHR LBD using *in silico* molecular modelling approaches, in order to understand differences and similarities among the three ligands.

2. Materials and methods

2.1. Chemicals

The test compounds IMA-06201 (C1; CAS Registry Number: 879410-94-3; Fig. 1) and IMA-06504 (C3; CAS: 1373259-57-4) were synthesized as described by Petterson (2012). The AHR antagonist CH-223191 (Kim, et al. 2006, Zhao, et al. 2010) was purchased from Sigma-Aldrich (St. Louis, MO USA), and TCDD from Ufa-Institute (Ufa, Russia; over 98% pure as assessed by gas chromatography–mass spectrometry). Solutions of all of the test compounds were prepared by dissolving in DMSO (Sigma-Aldrich). Dissolution of C1 and C3 stocks was aided by

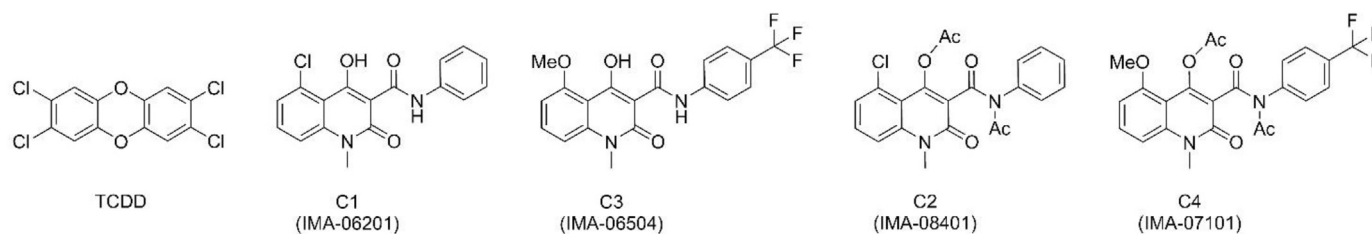


Fig. 1. Chemical structures of TCDD, the novel SAHRMs C1 and C3 used *in vitro*, and those of the respective, acetylated prodrugs C2 and C4 for *in vivo* use.

heating them in +37–65 °C or +65–85 °C waterbaths, respectively, for 30–60 min. For the H4IIE cells, all DMSO solutions were further diluted with cell culture medium before application to cells (final DMSO concentration on cells was 0.1%).

Chemicals used as positive controls included Triton X, benzo[a]pyrene (BaP), sodium azide (NaN₃), 2-aminoanthracene (2AA), and mitomycin C (all from Sigma). Apart from Triton X, they were dissolved in either H₂O or DMSO.

2.2. Cell culture

Rat hepatoma H4IIE cells, acquired from ATCC (H-4-II-E ATCC® CRL1548™; Manassas, VA USA), were used in all *in vitro* experiments, apart from the Ames test. This cell line was selected for its highly inducible expression of CYP xenobiotic-metabolising enzymes (Fujimura, et al. 2012). In addition, it has been shown to be exceptionally responsive to CYP1A1 induction by dioxins (Bradlaw and Casterline Jr. 1979, Sawyer and Safe 1982).

The cells were cultured at 37 °C and 5% CO₂ in low glucose EMEM (ATCC) or low glucose DMEM (Gibco® DMEM, Thermo Fisher Scientific, Paisley, Scotland, UK), depending on the medium used in the following experiment. Both media were supplemented with 10% FBS (Sigma-Aldrich) for cell culturing. Cell passages from 2 to 6 were used for the experiments. MycoAlert™ Mycoplasma Detection Kit (Lonza Group Ltd., Basel, Switzerland) was used periodically to ensure that the cells used in the experiments were free of infection.

2.3. LDH leakage assay

Cytotoxicity of C1, C3 and TCDD was assessed in H4IIE cells using Cytotoxicity Detection Kit (LDH; Roche Diagnostics GmbH, Mannheim, Germany), which measures lactate dehydrogenase (LDH) leakage. 25,000 cells were seeded into a well in 96-well plates (Greiner Bio-One GmbH, Kremsmünster, Austria) 18 h prior to exposures, except for the outer and corner wells which were only filled with PBS to avoid the edge effect. As recommended in the manual, medium (EMEM) supplemented with 1% FBS was used as assay medium to prevent LDH in sera from increasing background absorbance. The concentrations of the test compounds used were 10, 100, and 1000 nM for C1 and C3, and 10 and 100 nM for TCDD. Cells were exposed for 6 h and 24 h. Vehicle was used as negative control (0.1% DMSO), and Triton X-100 (1%) as positive control. Also background controls (assay medium without cells, with and without vehicle) were included. All exposures were performed in triplicate in two independent experiments. Culture supernatants were collected, and the assay performed according to the

manufacturer's instructions. Absorbances were measured with an ELISA reader at 492 and 620 nm (absorption values were corrected with background absorption; Multiskan Ascent, Thermo Fisher Scientific, Waltham, MA USA). Cytotoxicity was calculated as percent, using the following equation: $100 * (\text{exp. value} - \text{negative control}) / (\text{positive control} - \text{negative control})$. The negative control was thus at 0% cytotoxicity and the positive control at 100%, and the cytotoxicity of the test compounds expressed relative to them.

2.4. MTT reduction assay

The effect of C1, C3, and TCDD on cell viability/metabolic activity was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay in H4IIE cells. 24,000 cells were seeded per well in 96-well plates (Greiner Bio-One GmbH), 24 h prior to exposures. The outer and corner wells were only filled with PBS to avoid the edge effect. The medium used in the assay was low-glucose, phenol red free DMEM (Gibco® DMEM, Thermo Fisher Scientific, Paisley, Scotland, UK) supplemented with 4 mM L-Glutamine (Thermo Fisher Scientific), corresponding to the standard DMEM used for culturing the cells prior to the experiment. The medium used in the experiment was further supplemented with 1% FBS (Sigma-Aldrich). The cells were exposed to vehicle, C1, C3, or TCDD (all in DMSO, final concentration on cells 0.1%) for 6 h or 24 h at 5–6 concentrations (1, 10, 50, 100, 500, and 1000 nM C1 and C3 on cells; 1, 5, 10, 50, and 100 nM TCDD). All exposures were performed in triplicate in two independent experiments. After exposures to the test compounds, the cells were washed once with PBS, and treated with MTT for 5 h (Sigma-Aldrich; final concentration 1 mg/ml in cell culture medium). The medium was then gently removed, and formazan crystals dissolved in 200 µl of DMSO containing 0.1 M glycerine and 0.1 M NaCl. After incubation at room temperature for 10 min, the absorbances were measured with an ELISA reader at 595 nm (Multiskan Ascent, Thermo Fisher Scientific). The results were calculated as percentage over vehicle controls.

2.5. Ames test

Genotoxicity was first examined by the standard plate incorporation mutagenicity test, which was performed according to the principle of Maron and Ames (1983). Briefly, TA98 and TA100 *Salmonella* Typhimurium strains (Institut Pasteur, Paris, France) were used, and the test was performed both with and without metabolic activation, using 10% S9 SD rat liver mix (Trinova Biochem GmbH, Giessen, Germany). Directly prior to plating, master mixes were prepared for each exposure

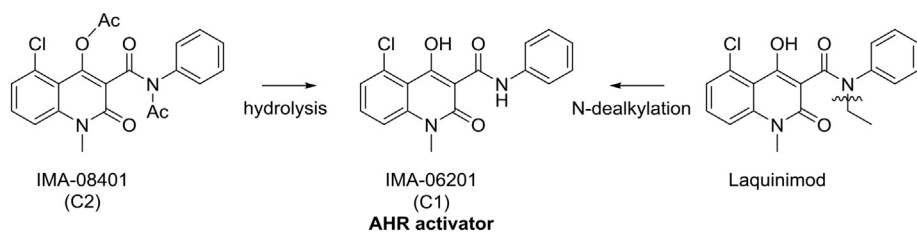


Fig. 2. The SAHRM C1 is formed *in vivo* by hydrolysis of the diacetate prodrug C2, but also in small amounts from the drug compound laquinimod by N-dealkylation. C3 is similarly formed *in vivo* from the prodrug C4 and the drug compound tasquinimod.

group, including (per plate): 50 µl bacteria, 100 µl histidine-biotine, 200 µl S9-mix (or NADPH-mix for plates without metabolic activation), and 50 µl test or control compound. The master mixes were mixed by pipetting, and 400 µl was added into each melted top agar (2.3 ml), vortexed briefly and poured onto a plate. Water and DMSO (1.85% on plates) were used as negative controls for both strains. As positive controls for TA100 and TA98, respectively, NaN_3 (0.04 mg/ml) or 2AA (0.2 mg/ml) were used. In addition, BaP (0.1 mg/ml) was used as a positive control for both strains. C1 and C3 were first tested at concentrations of 0.375, 0.75, 1.5 and 3 mg/ml, resulting in plate concentrations of ~17.5–170 µM. A repeat experiment was conducted at plate concentrations of 1 nM, 100 nM, 10 µM and 1 mM. The highest plate concentration of 1 mM can be considered the maximum achievable dose, as it precipitated when added to the master mix. All exposures were performed in triplicates in the first, and quadruplicates in the second experiment for each compound and dose, and for all controls. The plates were then incubated at 37 °C for 72 h before counting the colonies.

2.6. Micronucleus test

Genotoxicity of C1, C3, and TCDD was further assessed by the *in vitro* micronucleus test in H4IIE cells. 40,000 cells were seeded in 12-well plates (Greiner Bio-One GmbH), 24 h prior to exposures. The medium employed was low-glucose, phenol red free DMEM (Gibco® DMEM, Thermo Fisher Scientific) supplemented with 4 mM L-Glutamine (Thermo Fisher Scientific) and 10% FBS, corresponding to the standard DMEM used for culturing the cells prior to the experiment. The cells were exposed to C1, C3, and TCDD; mitomycin C as a positive control; and vehicle as negative control. Final concentrations on cells were: 100, 500, and 1000 nM for C1/C3; 10, 50, and 100 nM for TCDD; and 500 nM for mitomycin C. All of the compounds were dissolved in DMSO, its final concentration on cells was 0.1%. Apart from the vehicle control (where $n = 6$), all exposures were performed in triplicate in a single experiment. The exposure time was 24 h, after which the cells were treated with cytochalasin B (4 µg/ml; Cayman Chemical, Ann Arbor, MI USA) for 1.5–2 normal cell cycle lengths, ~28 h. Next, the cells were washed twice with PBS and fixed with 4% paraformaldehyde for 10–15 min (in PBS, pH 6.9; Sigma) before washing again with PBS. After the wells had dried, the cells were stained with 10% Giemsa solution (Merck KGaA, Darmstadt, Germany) and micronucleus frequencies analysed in 1000 binucleated cells per well.

2.7. CYP1A1 enzyme activity

CYP1A1 induction potential of C1, C3 and TCDD was assayed in the metabolically active H4IIE rat hepatoma cell line by a luminescent assay. CYP1A1 induction was utilised as a well-established, fairly rapid, and highly sensitive index of AHR activation (Abraham, et al. 1988). The aim was to compare the potency and efficacy of the novel compounds with those of TCDD, and to also test whether a specific AHR antagonist, CH-223191 (Kim, et al. 2006), is able to block any CYP1A1 induction. The experiments were performed in 96-well plates (Greiner Bio-One GmbH, Kremsmünster, Austria). Cells were seeded at 10,000 cells/well and allowed to equilibrate for about 40 h prior to exposures. The outer and corner wells were left without cells and filled with PBS in order to avoid the edge effect. The cells were then exposed for 48 h to varying concentrations of C1, C3 and TCDD (1, 10, 100, 500 pM and 1, 5, 10, 50, 100 nM, and 1000 nM for C1 and C3). Two independent experiments were carried out. In addition, to test whether the selective AHR-antagonist CH-223191 is able to block the effect of C1 in a similar manner to that of TCDD, cells were exposed for 24 h and 48 h to 1 nM C1 or TCDD, and a combination of 1 nM C1/TCDD and 100 nM CH-223191.

In all experiments, the controls were exposed to the vehicle (0.1% of DMSO in culture medium). For the 48 h treatments, culture medium

(EMEM + 10% FBS) with the test compounds was changed at 24 h and the exposures repeated. All exposures were performed in triplicates. After exposures, CYP1A1 activity was detected with P450-Glo™ CYP1A1 Assay (Promega, Madison, WI USA) according to manufacturer's instructions. Subsequently, CellTiter-Glo® Luminescent Cell Viability Assay (Promega) was used to confirm that there were no significant differences among the wells in their numbers of viable cells at the time of detection.

2.8. Molecular docking analysis

The three-dimensional structure of the rat AHR LBD was previously predicted (Motto, et al. 2011) by Homology Modelling using MODELLER (Webb and Sali 2016). The X-ray structures of the human HIF2α PAS-B domain in complex with different artificial ligands (PDB id: 3H82; 3F1O; 3H7W) were used as templates. The sequence alignment between the rat AHR (UniProt: P305615) and the template is reported in Supplementary Fig. 1. Representative conformations of the modelled LBD were selected among the one hundred models with the best DOPE score (Webb and Sali 2016) by cluster analysis and refined by the Schrödinger Protein Preparation Wizard (Schrödinger Release 2016–4: Protein Preparation Wizard, 2016).

The molecular structures of the C1 and C3 ligands were subjected to conformational analysis performed by *ab initio* Quantum Mechanical (QM) calculations at the RHF/6-31G* level, using Jaguar (Schrödinger Release 2016–4: Jaguar, 2016). The minimum energy conformations were obtained by systematic search of the torsional angle C4-C3-C11-O (Supplementary Fig. 2) with complete geometry optimization at every step (scan every 15°). Geometry optimization was carried out in water solution using a continuum solvent model.

Molecular Docking calculations were performed using Glide extra precision (XP; Schrödinger Release 2016–4: Glide, 2016). To include the part of the domain flexibility involved in ligand binding, different modelled conformations of the AHR LBD were used for docking (ensemble-docking technique), as previously described (Motto, et al. 2011), and a scaling factor of 0.8 was applied to the van der Waals radii of the protein atoms (soft-docking technique). In docking calculations, all the torsional angles of the ligands but the one associated to rotation of the phenyl group, were restrained according to the QM results and the QM net atomic charges were used for describing the ligand electron distribution. One binding geometry (pose) was obtained for each ligand in each modelled receptor conformation. To select the most favourable pose, the binding free energies (ΔG_{bind}) were calculated using the Molecular Mechanics Generalized Born Surface Area (MM-GBSA) approach implemented in Prime, which includes evaluation of the solvation effects by an implicit solvent model (Schrödinger Release 2016–4: Prime, 2016). Visualization of the models was accomplished using PyMOL (Schrödinger LLC. The PyMOL Molecular Graphics System).

2.9. Statistical analysis

Statistical analysis was performed applying the SPSS Statistics software (IBM SPSS Statistics for Windows, Version 24.0., Armonk, NY, USA) or GraphPad Prism (GraphPad Software, Inc., Prism 7 for Windows, Version 7.03, La Jolla, CA USA). Statistical significance was set at $p < .05$. Statistical analysis was mostly performed by one-way ANOVA in SPSS. All distributions were normal, but the variances proved to be non-homogeneous in some cases. However, both robust ANOVAs, Welch and Brown-Forsythe, indicated significant outcomes for all groups ($p < .001$), legitimating the approach. To compare each group against the vehicle controls, post-hoc analysis was performed by either Dunnett (when variances were homogenous) or Games-Howell test (in case variances were not homogenous). The data from the micronucleus test were statistically assessed by the Cochran-Armitage test for trend in proportions. Moreover, the dose-response data from the CYP1A1 activity assay was analysed in GraphPad Prism using nonlinear

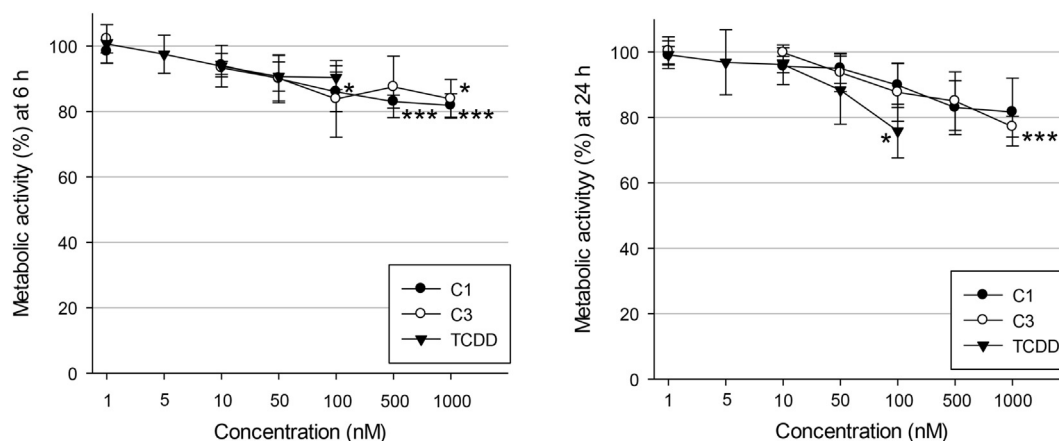


Fig. 3. The result of MTT assay by C1, C3, and TCDD at 6 h (left panel) and 24 h (right panel). The data are presented as percentage of vehicle control ($n = 6$; mean \pm SD). Groups that differ from the controls in a statistically significant manner are marked with an asterisk (* $p < .05$, *** $p \leq .001$). The single asterisk at 6 h, 100 nM, refers to C1.

regression with four-parameter logistics.

3. Results

3.1. Toxicity in vitro

3.1.1. LDH leakage assay

In the LDH leakage assay, at both 6 and 24 h time points, the maximum cytotoxicity caused by all three test compounds was only 5% at the concentrations tested (Supplementary Fig. 3; $n = 6$ /group). Furthermore, no dose-dependency was evident. In pairwise comparisons following one-way ANOVA ($p < .001$), the only group that differed from the others in a statistically significant manner ($p < .001$) was the positive control, 1% Triton X.

3.1.2. MTT reduction assay

A decrease in MTT reduction was observed by all three compounds, but at differing time points and concentrations (Fig. 3). At 6 h, multiple comparisons showed that for C1, the three highest concentrations (100, 500, and 1000 nM) differed significantly from the vehicle controls. C3 differed from the controls only at the highest concentration of 1000 nM. TCDD did not show a reducing effect at 6 h at any of the concentrations tested (1–100 nM).

At 24 h, a significant decrease in MTT reduction was seen with C3 likewise only at the highest concentration, while C1 did not induce a statistically significant effect at any concentration at this time point. With TCDD at 24 h, there was a statistically significant difference compared with controls at the highest concentration tested, 100 nM.

Interestingly, the effect of C1 and C3 as percentage of vehicle controls did not intensify dramatically between 6 h and 24 h. The maximum reduction percentages compared with controls were seen with C1 and C3 at 24 h and at 1000 nM, when, respectively, MTT reduction was down to 82% and 77% of controls. At 6 h, the respective percentages at 1000 nM were 82% and 84% of controls. Furthermore, between the 6- and 24-h time points, at concentrations up to 100 nM, the MTT reduction capacity of cells treated with C1 and C3 appeared to have even slightly recovered, showing systematically a slight lessening of reduction at 24 h compared with 6 h. The statistically significant reduction by TCDD observed at 24 h (down to 76% of controls at 100 nM of TCDD) was clearly more intense than the effect of C1 and C3 at 100 nM or even 500 nM at either time point.

3.1.3. Ames test

In the Ames test, C1 and C3 did not appear mutagenic at the concentrations tested (1 nM–1 mM) in either of the strains used, TA100 and

TA98. TCDD was not tested here. The number of revertants obtained with all tested concentrations of C1 and C3 were \leq ~1-fold of negative controls (complete data shown in Supplementary Tables 1. and 2.). The negative controls (DMSO and H₂O) performed consistently, and also positive controls performed adequately (NaN₃ and 2AA for TA100 and TA98, respectively, and BaP for both strains). The indirect mutagen BaP generated in some cases less revertants with metabolic activation than was expected, but all positive controls differed from the negative controls in a statistically significant manner.

In the TA100 strain, the numbers of revertants obtained with both C1 and C3, at all tested concentrations, and both with and without metabolic activation, were comparable to those of negative controls. The only test compound group that differed significantly from the negative controls in TA100 strain was C1, which at 10 μ M and in the absence of metabolic activation, appeared to be cytotoxic. This occurred only in the repeat experiment where it reduced the number of revertants, the average fold change over negative controls was 0.45. This effect was probably not treatment-related as it was not observed at any of the higher concentrations tested (altogether 5 concentration levels in the two experiments).

In the TA98 strain, both C1 and C3 appeared to be somewhat cytotoxic to the cells. In some cases in both experiments, the numbers of revertants obtained with C1 and C3 were considerably lower than with negative controls; however, not systematically. In the first experiment, with all concentrations tested (17.5–170 μ M) and with metabolic activation, the respective numbers of revertants with C3 and C1 were approximately 30–50% lower than with negative controls. In the first experiment, this effect was not observed without metabolic activation. However, in the repeat experiment, conducted with a larger concentration range, C1 appeared particularly cytotoxic without metabolic activation, but only at high concentrations; at 10 μ M and 1 mM, average fold changes over controls were 0.61 and 0.48, respectively. Furthermore, in the repeat experiment and with metabolic activation, the effect of C1 appeared less obvious, but there seemed to be a decreasing trend, the fold change at the highest concentration being 0.77. For C3 in TA98, cytotoxicity was seen at the highest concentration (1 mM) with and without metabolic activation, with the respective fold changes being 0.67 and 0.78.

3.1.4. Micronucleus test

The ability of C1 and C3 to augment micronucleus formation in the metabolically active H4IIE cells was used as a measure of their potential to elicit genotoxicity *in vitro*. For comparison, TCDD was tested simultaneously. The concentrations applied for C1 and C3 were 100, 500 and 1000 nM; for TCDD, they were 10-fold lower.

Table 1C1, C3, and TCDD were tested at three concentration levels in the *in vitro* micronucleus test. Mitomycin C was employed as a positive control.

Group	Conc (nM)	Micronuclei per 1000 binucleated cells			Average	SD	Fold	Trend
		Replicates						
Mitomycin C	500	147	162	161	157	7	10.44	
Vehicle control	0.1% DMSO	12	15	17				
C1	100	21	11	14	15	3	1.00	
	500	24	19	20	21	2	1.40	
	1000	25	22	24	24	1	1.58	$p = .083$
C3	100	28	31	23	27	3	1.82	
	500	25	22	30	26	3	1.71	
	1000	30	34	26	30	3	2.00	$p = .001$
TCDD	10	40	44	36	40	3	2.67	
	50	16	12	13	14	2	0.91	
	100	20	16	18	18	2	1.20	$p = .068$
		23	26	22	24	2	1.58	

Fold = Fold change vs. vehicle control.

In the case of TCDD and C1, a tendency towards increased frequency of micronuclei was discernible but did not quite reach statistical significance (Table 1). However, for C3 the Cochran-Armitage test of trend showed a statistically significant linear trend, $p = .001$, with the proportion cells containing micronuclei increasing as a function of concentration.

3.2. CYP1A1 induction potential

A luminescent assay was used to test C1, C3, and TCDD for their potential to induce CYP1A1, a sensitive marker for AHR activation, at 48 h. The metabolically active H4IIE cell line was used for the experiments, and the concentration range of the test compounds used was 0.001–100 nM (and 1000 nM for C1 and C3). In order to detect any changes in cell numbers, the number of viable cells in each well was examined at the end of every experiment by another luminescent assay. No significant differences in cell viability were observed with any of the compounds or concentrations.

In the CYP1A1 induction assay, both C1 and C3 were highly effective and potent activators of CYP1A1 (Fig. 4). The potencies of all three compounds were similar: compared with controls, C1 and C3 induced CYP1A1 significantly at concentrations of 100 pM and above ($p \leq .009$

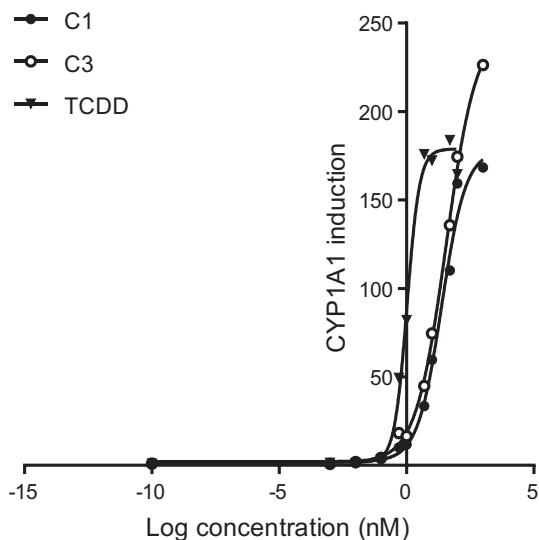


Fig. 4. CYP1A1 induction in H4IIE cells by C1, C3, and TCDD at the concentration range of 0.001–1000 nM (–100 nM for TCDD), expressed as fold changes over controls (mean, $n = 3$ –6/group; SDs are not presented here to improve readability, but are included in Supplementary Fig. 4).

for C1 and $p \leq .013$ for C3), and TCDD from 500 pM on ($p \leq .008$). However, TCDD was the most potent inducer of the three. At already 500 pM, the induction by TCDD was more intense than by C1 and C3, attaining a 50-fold difference compared with controls, while for C1 and C3 the respective fold changes were 10 and 20. The EC50 values for C1, C3, and TCDD were 24.1, 35.9, and 1.0 nM, respectively. The induction potential of TCDD peaked at 50 nM and appeared to level off after that. Interestingly, compared with TCDD, both C1 and C3 attained equivalent or even higher maximum induction of CYP1A1 at the highest concentration tested (1000 nM). Furthermore, it appears that the maximum responses with C1 and C3 may not have been reached at 1000 nM, and that C3, possibly also C1, have therefore even higher efficacies than TCDD.

Furthermore, we tested the ability of the selective AHR-antagonist CH-223191 to block CYP1A1 induction by C1 and TCDD with exposure times of 24 h and 48 h. At a concentration of 100 nM, CH-223191 was able to completely block the effect of 1 nM C1 and TCDD at both time points (Fig. 5).

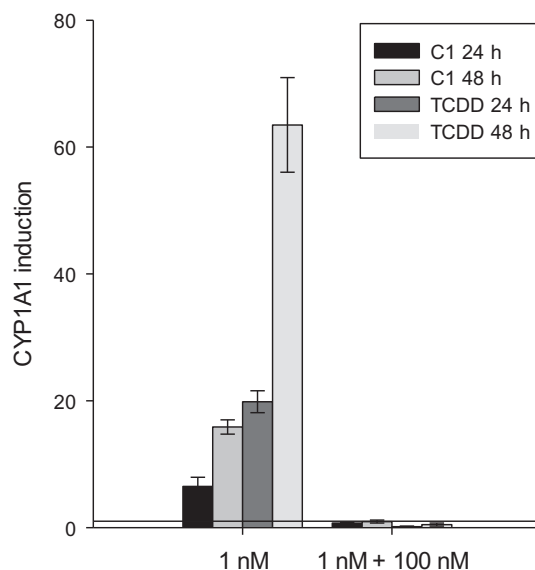


Fig. 5. CYP1A1 induction in H4IIE cells by 1 nM of C1 and TCDD, both without (at left) and with (at right) the presence of 100 nM of the AHR-antagonist CH-223191 ($n = 3$ /group). The exposure times were 24 h and 48 h for all treatments. The data are expressed as fold changes over controls, the reference line marks vehicle control level (fold change 1.0).

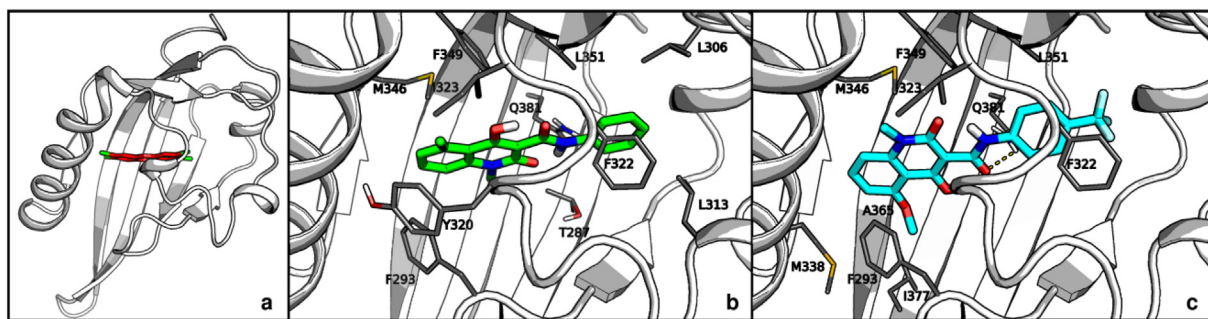


Fig. 6. Molecular Docking poses in the AHR model. a. The whole LBD in complex with the TCDD (red) inside the ligand binding cavity; b. The pose of C1 (green); c. The pose of C3 (cyan). Residues that mainly contribute to the pose stabilization are shown as lines and labelled. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Molecular docking analysis

The AHR LBD model is shown in Fig. 6a; it adopts the classical PAS fold with a β -sheet of 5 strands, 3 small helices and a long helical connector. The domain has a buried internal cavity that was proved to act as the TCDD binding region (Motto, et al. 2011, Pandini, et al. 2009).

Conformational analysis indicated a planar geometry for most of the molecular structure of both the C1 and C3 compounds, with a value of 0° for the examined torsional angle (Supplementary Fig. 2). The planar geometry can be attributed to an extended electron conjugation and to two stabilizing intra-molecular H-bonds. These results are consistent with the previous QM conformational analysis performed in vacuum by Jansson et al. (2006).

Molecular docking simulations confirmed that both C1 (Fig. 6b) and C3 (Fig. 6c) occupy the central region of the ligand binding cavity, similarly to what is predicted for the TCDD (Fig. 6a) in the high-affinity mouse and rat AHRs (Motto, et al. 2011). The ligand interactions that mainly contribute to stabilization of each pose, obtained by per-residue decomposition of the MM-GBSA ΔG_{bind} , involve residues at the centre of the cavity (F293, F322, I323, M346, F349, L351, Q381, shown in Fig. 6b and c). The polar groups of both ligands are involved in intra-molecular H-bonds, like in the unbound forms, so their stabilization within the binding cavity is mainly due to hydrophobic interactions (van der Waals, and aromatic stacking) with residue side-chains. C1 lies on the same lying plane as TCDD (Fig. 7a) and both ligands reach the most internal hydrophobic region (L306 and L313), TCDD through its chlorine atoms and C1 through its phenyl ring. C3 shows a slightly different lying plane (Fig. 7b) and the methoxy and the trifluoromethyl functional groups bring the molecule nearer the helical connector, thus translating the rest of the molecule forward compared to the other ligands. This slightly different placement allows the additional interactions of C3 with the M338 and I377 residues. The glutamine residue at the centre of the cavity (Q381) acts as an anchor providing inter-molecular H-bonds.

4. Discussion

While the term SAHRM has not been unambiguously defined, it is generally used to describe compounds that exhibit tissue- or species-specific AHR-modulation, or induce only some of the typical responses of AHR activation seen after dioxin exposure. SAHRMs may be naturally occurring compounds, or engineered with the aim of producing beneficial AHR-mediated effects, while lacking the toxic effects of many dioxins.

The interest in SAHRMs is in large part due to their potential as novel pharmacological compounds, but also because they can be valuable tools in further elucidating the so far incompetently understood, multifaceted physiological roles of AHR and the underlying molecular mechanisms. Currently, there are numerous compounds that are being studied as potential AHR-modulating drug compounds, primarily for treatment of different cancers and inflammatory diseases (Ehrlich and Kerkvliet 2017, Safe, et al. 2013, 2017). Among these compounds is laquinimod, which produces low but persistent levels of the active *N*-hydrogen metabolite C1. The mechanism of action of laquinimod is not yet fully elucidated, but it has been recognised as an immunomodulatory compound (Varrin-Doyer, et al. 2014), and the mode of action has been shown to be AHR dependent in the mouse experimental autoimmune encephalomyelitis (EAE) MS model (Berg, et al. 2016, European Medicines Agency 2014, Kaye, et al. 2016).

In humans, laquinimod is metabolised by CYP3A4 to hydroxylated and dealkylated metabolites (Tuvesson, et al. 2005). C1, also called DELAQ (de-ethylated laquinimod), is one of these metabolites, and has already previously been identified as a very potent AHR-activator (European Medicines Agency 2014, Pettersson 2012). Interestingly, C1 is also a more potent inhibitor of disease development in the EAE model than laquinimod (European Medicines Agency 2014). Furthermore, substantially higher levels of C1 are generated *in vivo* from the pro-drug C2 than from laquinimod (unpublished results). From laquinimod, for which the human $T_{1/2}$ is 71 h, C1 is continuously produced in low amounts, and further metabolised by hydroxylation in the aniline part *via* CYP1A-dependent metabolism (Tuvesson, et al. 2005). *In vitro* clearance data (human hepatic extraction 84%, unpublished) and *in vivo* pharmacokinetic data indicate that after C2 administration, C1 is

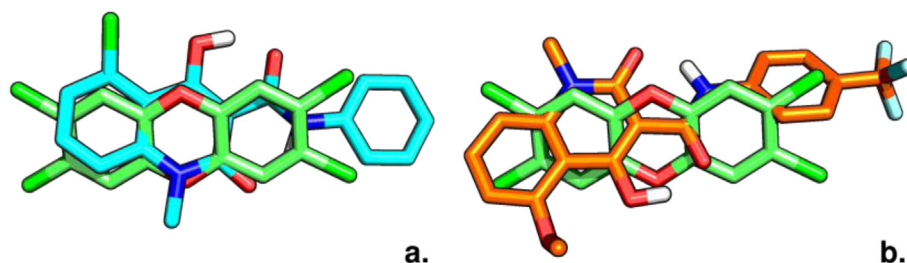


Fig. 7. Comparison between the TCDD docked pose and the poses of C1 (a) and C3 (b).

degraded in a fairly rapid manner (T_{1/2} in rats 1.7 h, unpublished). Thus, administration of the prodrug C2, with efficient hydrolysis to C1, produces quite a different exposure profile compared with laquinimod administration, with temporal but higher levels of C1, avoiding prolonged and aberrant AHR activation.

In this study, we characterised the *in vitro* effects and *in silico* binding of two novel SAHRMs, C1 and C3. Their respective prodrugs, C2 and C4, were previously shown to be markedly less toxic than TCDD in SD rats, while being effective AHR activators (Mahiout, et al. 2017). Intriguingly, all of the dioxin-like effects observed *in vivo* fell into the “type I” category previously demonstrated to be largely indifferent to structural variation at the transactivation domain of the AHR in TCDD-treated rats (Pohjanvirta, et al. 2011). Thus, these compounds may also have wider implications to AHR research.

Here, we examined C1 and C3 *in vitro* for their cytotoxicity by LDH leakage assay, their effect on metabolic activity by MTT reduction assay, and their genotoxic potential by Ames and micronucleus tests. In addition, we tested their AHR-inducing potential, interpreted from activation of the xenobiotic-metabolising enzyme CYP1A1, a sensitive biomarker for AHR activation (Abraham, et al. 1988). Finally, we compared the binding of C1 and C3 to the rat AHR *in silico*, and compared them with TCDD, the most toxic AHR activator.

In the LDH leakage assay, C1, C3, and TCDD were tested at 2–3 concentrations and at 6 h and 24 h exposure periods in H4IIE cells. Both negative (vehicle) and positive controls (1% Triton X) were included, and the cytotoxicity of the three test compounds expressed relative to them. None of the test compounds exhibited cytotoxicity at the tested concentrations (10 and 100 nM for TCDD, and also 1000 nM for C1 and C3) at either of the time points. However, while being a robust method and widely used, the LDH leakage assay only detects cytotoxicity following damage to the cell membrane, as LDH is released from the cytosol. Therefore, it does not identify compounds that cause cytotoxicity via other pathways. Thus, we also tested C1, C3, and TCDD by MTT reduction assay.

In the MTT assay we examined the effect of C1, C3, and TCDD on H4IIE cells at 5–6 concentrations (1–1000 nM, for TCDD the maximum concentration was 100 nM) at 6 h and 24 h exposure periods; also vehicle controls were included. The colorimetric MTT assay is based on the principle that viable cells with active metabolism convert the yellow tetrazolium dye MTT into a purple formazan product, which can be measured. When cells die, they can no longer make this conversion. The molecular pathways involved in disturbances in MTT reduction into formazan are not well understood, but in controlled study conditions, the amount of formazan product formed by NAD(P)H-dependent cellular oxidoreductase enzymes is in proportion to the number of metabolically active, viable cells (Riss et al., 2013 (Updated 2016)). Therefore, and as the data from the test compounds were compared with vehicle controls (100% viability and metabolic activity), the result was interpreted to reflect a reduction in metabolic activity.

A reduction was observed by all three compounds (Fig. 3). However, only C1 and C3 induced an effect at 6 h, when statistical analysis revealed a significant effect for C1 at the three highest concentrations starting from 100 nM, and for C3 at the highest concentration of 1000 nM. Therefore at 6 h, C1 and C3 appeared to reduce metabolic activation more effectively than TCDD. Interestingly at 24 h, statistically significant reductions were not seen with C1 at any of the concentrations tested, and also with C3 only at the highest concentration of 1000 nM. Furthermore, between the 6- and 24-h time points, the MTT reduction capacity of the cells treated with C1 and C3 (up to concentrations of 100 nM), appeared to have even slightly recovered. Moreover at 24 h, TCDD induced a statistically significant reduction already at 100 nM. It therefore appeared to reduce metabolic activation at 24 h ~10-fold more effectively than C3. It should be noted that despite the small differences in the concentrations at which the effects of C1 and C3 reached statistical significance, overall their impacts were highly similar.

Taken together, these data on MTT reduction support the hypothesis we presented before (Mahiout, et al. 2017) that C1 and C3 are transformed to inactive metabolites much more efficiently than TCDD, likely at least partly explaining their considerably lower toxicity *in vivo*.

In the Ames test, the *Salmonella typhimurium* strains used were TA98 and TA100, which contain the *hisD3052* and *hisG46* mutations, respectively (Maron and Ames 1983). The TA98 strain primarily detects mutagens that cause frameshift mutations, while the TA100 preferentially detects mutagens causing base-pair substitutions. Only C1 and C3 were tested here, as it has been previously shown that TCDD is not mutagenic (Thornton, et al. 2001).

Neither of the compounds appeared mutagenic at the concentration range tested: 1 nM–1 mM. The positive controls performed adequately overall and all of them produced statistically significant positive results compared with negative controls. However, in some cases the indirect mutagen BaP yielded rather low numbers of revertants with metabolic activation, indicating that the microsomal S9 mix did not work as efficiently as desired.

Some cytotoxicity was observed in the TA98 strain. The only systematic dose-responses in this respect were observed with metabolic activation by C1 at or above ~20 μM, and by C3 at or above ~70 μM. Moreover, cytotoxicity also manifested without metabolic activation at the highest concentration of 1 mM by both C1 and C3. Furthermore, the plate concentrations of 1 mM can be considered the maximum achievable, as already at those both C1 and C3 precipitated when added to the master mix prior to plating.

Therefore, despite some limitations in the data, the negative result of the Ames test can be considered reliable for both compounds, particularly in the TA100 strain. It thus appears that in the Ames test, C1 and C3 do not induce base-pair substitutions, and are unlikely to induce frameshift mutations. However, as the Ames test is only able to detect mutagens, we also performed an *in vitro* micronucleus test on the H4IIE cell line to further assess the potential for genotoxicity of these compounds. S9 mix was not employed, as the cell line has retained considerably high metabolic activity (Fujimura, et al. 2012). In the micronucleus assay, C3 induced a statistically significant, increasing trend in number of micronuclei, while C1 and TCDD showed a tendency for statistical significance (Table 1). However, the significance of the positive effect by C3 is unclear, particularly considering the small magnitude of the fold-change vs. the positive control (mitomycin C) and the similar result obtained with TCDD.

TCDD is a carcinogen, apparently as a consequence of several complex pathways related to, at least, tumour promotion (Pitot, et al. 1980), modulation of genes related to cell proliferation and immune system (Bersten, et al. 2013), and regulation of apoptosis (Schrenk and Chopra 2011). However, TCDD is not a genotoxicant *in vivo* (Huff, et al. 1991, Meyne, et al. 1985, Nebert, et al. 2004), even though particularly *in vitro*, it has occasionally appeared positive at high concentrations in genotoxicity tests, including a micronucleus test in rat hepatocytes (Turkez, et al. 2014). TCDD is known to cause oxidative stress (Stohs 1990), and oxidative stress may secondarily augment micronucleus formation *in vitro* (Cicchetti and Argentin 2003). Therefore, it is conceivable that oxidative stress is also involved in the increased micronucleus frequency found for C3 in the present study. However *in vivo*, following AHR-activation by AHR-agonists, subsequent induction of metabolic enzymes, particularly CYP1A1, appears to have protective effects due to augmented detoxification capacity, unlike *in vitro* (Nebert, et al. 2004). Therefore, in order to conclude whether the effect seen here with C3 is relevant, an *in vivo* micronucleus test may be necessary.

In order to study the potency and efficacy of these compounds as AHR-activators, we had previously screened CYP1A1 induction potentials of C1, C3, and TCDD after 24-h exposures, employing the same H4IIE cell line and concentrations of 1–50 nM (Mahiout, et al. 2017). There, both compounds, and particularly C3, induced responses close to the same fold-range as TCDD. The result here, at 48 h exposure and also

at higher concentrations of each of the compounds, was comparable. This study also confirmed that C3 appears to be the (somewhat) more effective compound of the two, as assessed by CYP1A1 induction. A possible reason for this difference may be a higher metabolic stability of C3, which is protected from para-hydroxylation in the aniline part by the CF₃-group, especially under conditions where CYP1A is upregulated.

Moreover, both novel SAHRMs, C1 and C3, were here shown to be similar to TCDD in both potency and efficacy. However, when comparing potencies and efficacies, a confounding factor may be the extremely low aqueous solubility of the compounds. Although the aqueous solubilities of both C1 and C3 are very low, estimated at < 1 mg/l (approximately 3 μM), this is conceivably higher than that of TCDD (< 1 nM; (Marple, et al. 1986). CYP1A1 induction may be dampened at concentrations exceeding the aqueous solubility of each compound. Another factor that can influence the activity of C1 and C3, respectively, is the aforementioned metabolic stability, which may favour the more stable C3.

These results are furthermore supported by the *in vivo* data with these compounds. In SD rats, we previously showed that C2, the pro-drug that is readily hydrolysed into C1 *in vivo*, is an effective inducer of the *Cyp1a1* gene at doses of 4, 20, and 100 mg/kg. Even the lowest dose tested, 4 mg/kg administered as a single ig dose, induced *Cyp1a1* induction by 1700-fold at 28 h after exposure, a strong early response compared with the 350-fold increase brought about by 0.1 mg/kg TCDD at 24 h (Mahiout and Pohjanvirta 2016). However, while the response with TCDD intensifies over time, it appears that C2 is metabolised rapidly: after single doses of 20 and 100 mg/kg, *Cyp1a1* induction fold changes at 48 h and 72 h, respectively, were down to a ~100 fold increase compared with controls. The MTT reduction assay results presented here further support the view that C1 and C3 are inactivated by metabolism much more rapidly than TCDD.

Furthermore, we showed here that the AHR-antagonist CH-223191, reported to be a selective antagonist of only dioxin-like AHR activators (Kim, et al. 2006, Zhao, et al. 2010), was able to block CYP1A1 induction by C1 *in vitro* as efficiently as that of TCDD. This indicates that at least C1 may bind to the LBD of the AHR in a manner similar to that of TCDD.

We also studied the ability of C1 and C3 to bind the AHR by *in silico* methods. Simulation of ligand binding by molecular docking to the homology model of the AHR LBD allowed prediction of both their binding geometries and protein-ligand interactions to be similar to those of TCDD (Fig. 6). In the docked poses, C1 and C3 show mostly planar conformations like TCDD, thanks to the intra-molecular H-bonds. TCDD and C1 binding poses are overlapped, especially in the region of the aromatic rings, whereas the C3 pose is slightly translated (Fig. 7). Despite this subtle difference, most of the stabilizing interactions are maintained in the two ligands and cause similar binding affinities for the AHR. Moreover, most of the residues that were predicted to be involved in the C1 and C3 stabilization (Fig. 6) belong to the group of highly conserved residues lining the binding cavities of several mammalian AHRs and necessary for optimal TCDD binding (“TCDD binding-fingerprint”) (Motto, et al. 2011, Pandini, et al. 2009). Therefore, our computational results support the hypothesis that these novel SAHRM metabolites effectively bind to the AHR and act as its agonists.

Interestingly, a previous study found that, based on their interactions with certain amino acid residues in AHR ligand-binding domain, diverse AHR ligands could be categorized into four groups, one of which comprised TCDD and other compounds sharing the dioxin-like toxicity profile (Soshilov and Denison 2014). While this outcome could be interpreted to suggest that the binding characteristics of a ligand to the AHR LBD would be critical to its toxicity properties, our present findings do not support this. However, the segregation was accomplished by mutating individual amino acids of the LBD, and only by an identical approach could it be verified whether our novel SAHRMs indeed are at odds with the classification. Although the persistence of

AHR-dependent gene expression generated by metabolically stable TCDD-like halogenated compounds is established to be crucial for the prototypical spectrum of AHR-dependent toxicities (Bradshaw and Bell 2009), attempts to reproduce the toxicity profile of TCDD by repeated exposure to huge doses of less stable AHR agonists have consistently failed (Francis and Smith 1987, Neal, et al. 1979). Hence, other factors are likely to contribute. Nevertheless, the rate of metabolism probably plays a major part in the case of differences in toxicity between TCDD and C1 & C3, whereas ligand binding is of low importance in this regard.

Altogether, these results establish that while showing negligible *in vitro* toxicity, the novel SAHRMs C1 and C3 bind to the AHR in a manner similar to that of TCDD, and are potent and effective AHR-agonists, in fact comparable to TCDD. Combined with our earlier results demonstrating that they seem considerably less toxic *in vivo* than TCDD (Mahiout, et al. 2017), these SAHRMs appear interesting candidates for therapeutic uses, and could also be valuable tools in further elucidating the multifaceted physiological roles of the AHR, and the underlying molecular mechanisms.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.tiv.2018.06.010>.

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