Supplementary Information SI4

The Effect of the Ratios [Exogenous AHR/Endogenous AHR] and [Ancient EC_{50}/Modern EC_{50}] on the Dose-Response Relation of TCDD-Activated CYP1A1 Induction.

Introduction

The HeLa human cervix epithelial adenocarcinoma cell line has been reported (Koyano et al. 2005) to express low background levels of endogenous *AHR* mRNA and protein and was therefore found suitable by the authors and reviewers of this paper for testing genetic *AHR* variants by transfection with an expression vector for the variant under study. Nevertheless, we chose to re-evaluate the *AHR* mRNA background level in the specific HeLa cell strain used in this study. We found a certain background for *AHR*, but less than in the prototypical AHR-responsive human liver cell line HepG2, and about 2 times higher levels for the AHR dimerization partner *ARNT* (Fig. SI4.1).

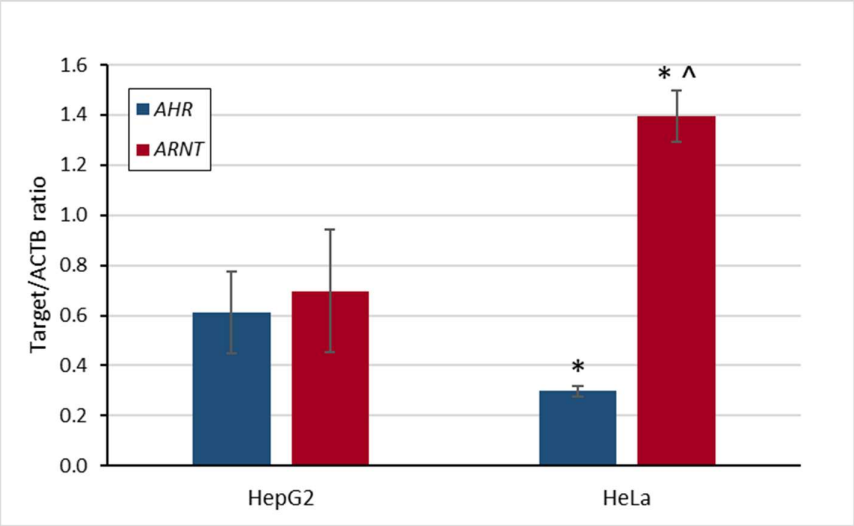

Fig. SI4.1. *AHR* and *ARNT* mRNA expression levels in AHR-deficient HeLa cells as compared to the notoriously AHR-competent HepG2 human liver carcinoma cell line. mRNA expression was measured by qRT-PCR using the *AHR* and *ARNT* primers listed in Supplementary Information Table SI8. Data are the mean of three (including two different harvesting dates) and six biological replicates for HepG2 and HeLa cells, respectively. Statistical significance of the difference observed between HeLa cells and the HepG2 reference was determined using Student's t-test: * = $p < 0.1$; ^ = $p < 0.05$.

The maximal CYP1A1 induction level elicited by the endogenous amount of AHR in HeLa cells attained up to 50% of the maximal CYP1A1 induction level observed upon transfection with an exogenous modern human or Neanderthal AHR expression construct (Fig. SI4.2).

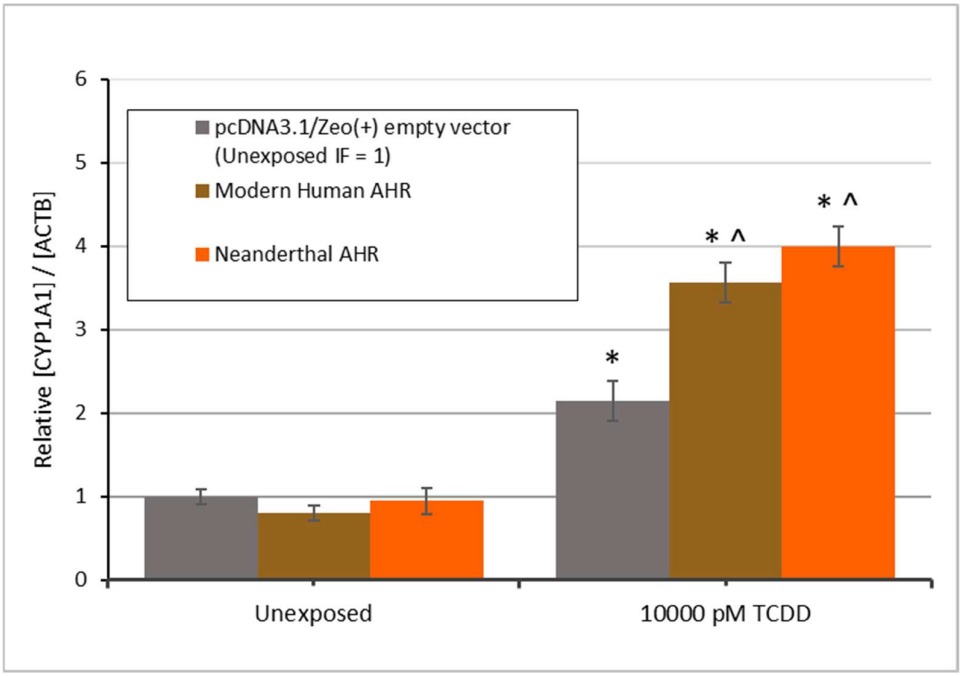

Fig. SI4.2. Maximal CYP1A1 induction level as observed upon exposure of HeLa human cervix epithelial adenocarcinoma cells to 10000 pM of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) when, respectively, transfected with an "empty" pcDNA3.1Zeo(+) expression construct (without any insert), or the same expression vector carrying a modern human or Neanderthal *AHR* cDNA sequence inserted downstream of the human cytomegalovirus (CMV) immediate-early promoter/enhancer present in the pcDNA3.1/Zeo(+) backbone. Three biological replicates were exposed 24 hours to a saturating concentration (10000 pM) of TCDD or dimethyl sulfoxide (DMSO) vehicle (Unexposed), and CYP1A1 mRNA measurements were carried out by qRT-PCR as described in the main text using the primers listed in Table SI8. The CYP1A1 mRNA induction responses were normalized to the average response of the unexposed empty vector control, *i.e.* its CYP1A1 expression was considered as the relative level with induction factor (IF) = 1. A significant difference according to Student's t-test ($p < 0.05$) with the corresponding unexposed transfected cells (*) or with the empty vector-transfected cells exposed to 10000 pM TCDD (^) is indicated.

Quantification of the *AHR* mRNA levels attained upon transfection with modern human and Neanderthal AHR expression constructs showed, however, that the AHR overexpression is much higher than the factor of 2 suggested by the empty vector control experiments, ranging from 17 - 141 times (Fig. SI4.3). The measured overexpression level of individual wells can vary, mostly within the same range for the modern human and Neanderthal AHR expression construct, differing by a factor 2 - 3, although two relatively high values were noticed for the modern human. Despite this, the difference in mean overexpression is not statistically significant ($p < 0.152$). We did not observe an effect of the overexpression variation range reported here on the CYP1A1 induction, probably due to the "spare receptor" effect previously described for the AHR (Greenlee and Poland 1979; Hestermann et al. 2000). This is also confirmed by the mathematical modeling of the effect of the exogenous AHR overexpression level on CYP1A1 expression transactivation (see below).

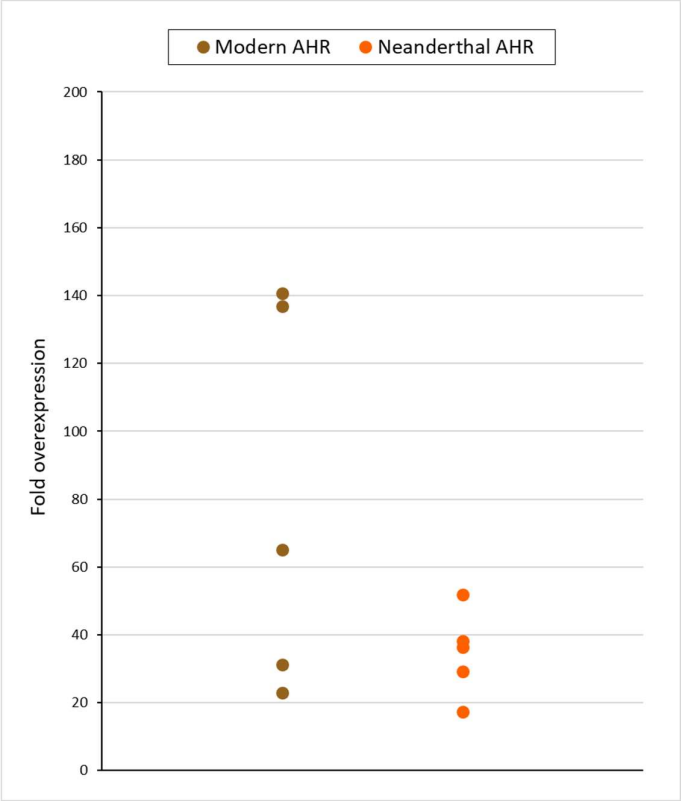

Fig. SI4.3. *AHR* mRNA overexpression in HeLa cells transfected with pcDNA3.1/Zeo(+)-based AHR expression constructs as compared to (endogenous) *AHR* mRNA levels in HeLa cells transfected with pcDNA3.1/Zeo(+) without insert (fold overexpression = 1). Scatter graph presenting the distribution of the data (n = 5).

The observed high level of overexpression is very likely to result in a far higher participation of the exogenous, transfected AHR in the measured CYP1A1 induction response than 50%. It is however experimentally very difficult to measure the relative participation of the endogenous and exogenous AHR in AHR-regulated responses. Therefore, we performed mathematical modeling of the effect of the exogenously / endogenously expressed AHR protein ratio on transactivation of CYP1A1 expression (replaced by its equivalent endogenous / total AHR ratio to reduce mathematical complexity).

Mathematical Modeling

Using the known computational relationship (Equation 1) between the concentration of a full AHR agonist, such as TCDD, and an AHR-controlled response, such as CYP1A1 induction, enables to predict the effect on the dose-response relation of the endogenous to total AHR ratio (R) and the Neanderthal to modern human EC_{50} ratio (F) (Equations 2 to 7).

$$\mathbf{Y = Bottom + [X * (Top\text{-}Bottom) / (EC_{50} + X)]} \qquad \text{(Equation 1)}$$

Y = CYP1A1 mRNA level
X = Concentration TCDD
Bottom = Basal CYP1A1 level
Top = Maximal CYP1A1 induction level
EC_{50} = Effective Concentration 50% (X-value producing a half-maximal response)

Since there is no a priori reason to presume interactive transactivation behavior of the endogenous and exogenous AHR molecules, we have assumed additivity between the response elicited by the endogenous and exogenous AHR in the model tested (Table SI4.1).

Table SI4.1. Mathematical model of the total CYP1A1 induction response caused by the additive action of agonist-activated endogenous and exogenous AHR in dependence of the $AHR_{Endogenous}$ / AHR_{Total} and the $EC50_{Neanderthal}$ / $EC50_{Modern\ human}$ ratios. Abbreviations used: Endo = Endogenous; Exo = Exogenous; Modern = Modern human; Nea = Neanderthal.

$$\mathbf{Y_{Total} = Y_{Endo} + Y_{Exo}} \qquad \text{(Equation 2)}$$

F is the factorial difference between the agonist responsiveness of the modern human and Neanderthal AHRs, as defined by the equation below:

$$\mathbf{EC50_{Nea} = F * EC50_{Modern}} \qquad \text{(Equation 3)}$$

R is the ratio of the amount of endogenous AHR to the total amount of AHR**:**

$$\mathbf{R = AHR_{Endo} / AHR_{Total}} \qquad \text{(Equation 4)}$$

or in terms of AHR_{Exo} excess: $R = [(AHR_{Exo} / AHR_{Endo}) + 1]^{-1}$ (Equation 4a)

If transfected with a pcDNA3.1/Zeo(+)-[modern human AHR] expression construct:

$$\mathbf{Y_{Endo} = (Bottom_{Modern} * R) + [X * (Top_{Modern} - Bottom_{Modern}) * R / (EC50_{Modern} + X)]} \qquad \text{(Equation 5)}$$

$$\mathbf{Y_{Exo} = [Bottom_{Modern} * (1 - R)] + [X * (Top_{Modern} - Bottom_{Modern}) * (1 - R) / (EC50_{Modern} + X)]} \qquad \text{(Equation 6)}$$

If transfected with a pcDNA3.1/Zeo(+)-[Neanderthal AHR] expression construct:

$$\mathbf{Y_{Endo} = (Bottom_{Modern} * R) + [X * (Top_{Modern} - Bottom_{Modern}) * R / (EC50_{Modern} + X)]} \qquad \text{(Equation 5)}$$

$$\mathbf{Y_{Exo} = [Bottom_{Nea} * (1 - R)] + \{X * (Top_{Nea} - Bottom_{Nea}) * (1 - R) / [(F * EC50_{Modern}) + X]\}} \qquad \text{(Equation 7)}$$

The values of the parameters $Top_{Modern\ human}$, $Top_{Neanderthal}$, $Bottom_{Modern\ human}$, $Bottom_{Neanderthal}$, and $EC50_{Modern\ human}$ were set at realistic values for a human cellular environment, *i.e.* the values as observed in human HeLa cells in this study (see Table 1 in the main text).

This additive model implies that the value of R will drop out of the equation for $Y_{Total} = Y_{Endogenous} + Y_{Exogenous}$ in case of transfection with a modern human AHR expression construct (SI5), and will not affect the dose-response relationship predicted. Therefore, the figures below do not show the predicted dose-response curve for transfection with a modern human AHR expression construct, since this curve will always coincide with the observed dose-response curve, irrespective of the value of R.

Fig. SI4.4, panels a-c, show the dose-response relationship for CYP1A1 mRNA induction by TCDD upon transfection with the Neanderthal *AHR* variant at various values for R (= endogenous / total AHR) and F (= Neanderthal / Modern Human EC_{50} ratio) as predicted by the mathematical model tested (Equations 1 - 7). The calculation of the data points is provided in Supplementary Information SI5.

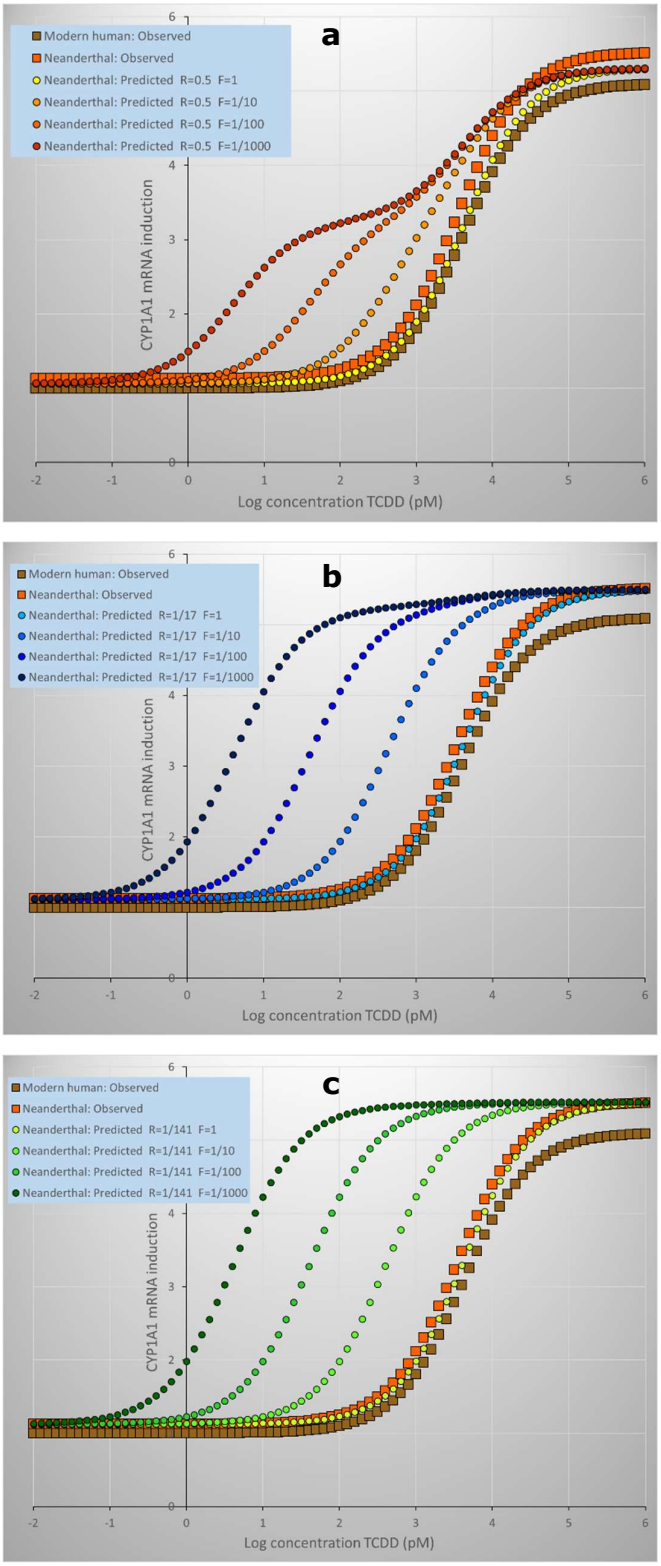

Fig. SI4.4. Comparison between the observed and the predicted dose-response relation for CYP1A1 mRNA induction by TCDD upon transfection of HeLa cells with an AHR expression construct when **(panel a)**, in the extreme case, the endogenous to exogenous AHR ratio in AHR-transfected cells would be 50%/50% (R=0.5); and when **(panel b)** the total over endogenous AHR excess in AHR-transfected cells would be 17-fold (R=1/17), being the lower limit, respectively **(panel c)**, 141-fold (R=1/141), being the upper limit of the AHR overexpression range observed in this study. Four different values of F (Neanderthal/Modern human EC_{50} ratio) were tested, representing no (significant) difference between the EC_{50} of the modern human and Neanderthal AHR (F=1), as in our study, and F=1/10, F=1/100, and F=1/1000, covering an increasing EC_{50} difference up to 1000-fold.

Fig. SI4.4a presents the mathematical analysis if the exogenous amount of AHR would not exceed, but just equal the endogenous amount ($AHR_{Endogenous}$ / AHR_{Total} = R = 0.5):

1. Most striking, but not surprising if there is no dominance of the exogenous over the endogenous AHR molecules, is that the dose-response relation is predicted to be essentially biphasic if F < 1, and hence the EC_{50} of the exogenous Neanderthal AHR is lower than the EC_{50} of the endogenous modern human AHR. This is reflecting that the Neanderthal AHR becomes already activated at lower TCDD concentrations than the modern human AHR.
2. This biphasic characteristic becomes more prominent with an increasing difference in EC_{50} between the Neanderthal and modern human AHR (the more F deviates from 1).
3. The maximal induction response predicted by the model is intermediate between the maxima observed for the modern human and Neanderthal AHR-transfected cells, and appears to be independent of the value of F.
4. Even in this extreme case, when there is supposed to be no excess of exogenous over endogenous AHR molecules, the Neanderthal AHR dose-response curve is predicted to be displaced about one order of magnitude or more towards the lower TCDD concentration range as compared to the curve of the modern human AHR, if F deviates substantially from 1 (F = 1/10, F = 1/100, F = 1/1000).

Fig SI4.4b presents the mathematical analysis in case of a 16-fold excess of exogenous over endogenous AHR upon transfection ($AHR_{Endogenous}$ / AHR_{Total} = R = 1/17), representing the lower limit of overexpression measured in this study:

1. Because of the excess of exogenous over endogenous AHR molecules, the contribution of the endogenous receptor to the total response is expected to be diminished. This explains why the model indicates that the biphasic character of the dose-response relation becomes suppressed as compared to R = 0.5.
2. Even at the measured lower limit of 16-fold excess of exogenous AHR, the biphasic suppression appears almost complete, so that a very slight biphasic pattern is only visible in case F = 1/1000; when F = 1/100 and F = 1/10 the biphasic behavior becomes indistinguishable. If F = 1/1000, the second phase of the curve begins when the endogenous modern human receptor starts to become activated as well, essentially at 1000 times higher TCDD concentrations than required for activation of the Neanderthal AHR. However, due to the excess of exogenous, highly responsive Neanderthal AHR, the total response is almost at its maximal level already in this high TCDD concentration range.

Due to the excess of exogenous Neanderthal over endogenous modern human AHR as compared to R = 0.5 the following can be observed:

3. The maximal induction response predicted by the model shifts towards the maximum observed for the Neanderthal AHR-transfected cells, irrespective of the value of F.
4. The shift along the x-axis of the Neanderthal AHR dose-response curve towards lower TCDD concentrations, occurring if F deviates substantially from 1 (F = 1/10, F = 1/100, F = 1/1000), becomes even more prominent.

Fig. SI4.4c presents the mathematical analysis in case of a 140-fold excess of exogenous over endogenous AHR upon transfection ($AHR_{Endogenous}$ / AHR_{Total} = R = 1/141), representing the upper limit of overexpression measured in this study:

1. Due to the greater 140-fold excess of exogenous over endogenous AHR molecules, the contribution of the endogenous receptor to the total response is expected to diminish even more.

2. Therefore, the biphasic dose-response behavior is practically cancelled out and becomes totally imperceptible.

Due to the even greater excess of exogenous Neanderthal over endogenous modern human AHR as compared to R = 1/17 the following can be observed:

3. The maximal induction response predicted by the model practically coincides with the maximum observed for the Neanderthal AHR-transfected cells, irrespective of the value of F.
4. The shift along the x-axis of the Neanderthal AHR dose-response curve towards lower TCDD concentrations if F < 1 is hardly affected, and practically occurs to the same extent when R = 1/141 as when R = 1/17.

Concluding Remarks

1. Fig. SI4.4a-c clearly support that a shift of the dose-response curve towards lower TCDD concentrations would have occurred in our experimental set-up, if a distinct difference in EC50 between modern human and Neanderthal AHR would have existed, irrespective of the value of R within the tested range (R = 0.5 – 1/141).
2. Even in the event that the exogenous AHR levels attained upon transfection would be about equal to the endogenous level (R = 0.5), which represents the "worst case" given the result of the empty vector control (Fig. SI4.2), any significant difference between the modern human and Neanderthal AHR in EC_{50} would still be clearly detectable. Fig. SI4.4a shows that the limit of detection would still lay substantially below a factor of 10 difference (confer F = 1 to F = 1/10 in Fig. SI4.4a).
3. The coincidence of a large difference in EC_{50} values (as reported by Hubbard et al. (2016)) with a low excess of exogenous over endogenous AHR can be excluded, since this combination would have resulted in a biphasic dose-response curve (Fig. SI4.4a), which we clearly did not observe.
4. The fact that the observed dose-response curves do almost coincide with the curves predicted by this model for the actual case (F = 1 and R << 0.5) is supporting that the assumption of additivity regarding the contribution of the exogenous and endogenous AHR to the total CYP1A1 induction is a reasonably truthful approximation of the actual process inside cells.

References

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