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Epigenetic Modifications Control CYP1A1 Inducibility in Human and Rat Keratinocytes

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Running Title: Transcriptional effects of epigenetic modulators on TCDD-elicited gene expression

Key words: Aryl hydrocarbon receptor, cycloheximide, HDAC inhibitors, superinduction, TCDD

Abstract

Serially passaged rat keratinocytes exhibit dramatically attenuated induction of *Cyp1a1* by aryl hydrocarbon receptor ligands such as TCDD. However, the sensitivity to induction can be restored by protein synthesis inhibition. Previous work revealed that the functionality of the receptor was not affected by passaging. The present work explored the possibility of epigenetic silencing on CYP1A1 inducibility in both rat and human cells. Use of an array of small molecule epigenetic modulators demonstrated that inhibition of histone deacetylases mimicked the effect of protein synthesis inhibition. Consistent with this finding, cycloheximide treatment also reduced histone deacetylase activity. More importantly, when compared to human *CYP1A1*, rat *Cyp1a1* exhibited much greater sensitivity toward epigenetic modulators, particularly inhibitors of histone deacetylases. Other genes in the aryl hydrocarbon receptor domain showed variable and less dramatic responses to histone deacetylase inhibitors. These findings highlight a potential species difference in epigenetics that must be considered when extrapolating results from rodent models to humans and has implications for xenobiotic- or drug-drug interactions where CYP1A1 activity plays an important role.

Introduction

The aryl hydrocarbon receptor (AHR) is a ligand-dependent transcription factor that responds to various exogenous and endogenous stimulants. By regulating a battery of genes, AHR can produce a diverse spectrum of biological and toxicological outcomes in a wide range of tissues and species (Denison et al. 2011). One of the best characterized high-affinity AHR ligands is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a prototypical halogenated aromatic hydrocarbon (DeGroot et al. 2011). Activation of the AHR by TCDD can elicit a robust transcriptional change at the target gene cytochrome P450 1A1 (CYP1A1), a phase I metabolizing enzyme (Whitlock 1999). Studies on TCDD-induced CYP1A1 expression have therefore permitted major mechanistic understanding of AHR signaling. In addition to CYP1A1, AHR also regulates the expression of other metabolizing enzymes, such as CYP1B1 (Sutter et al. 1994), GSTA1 (Rushmore et al. 1990), and ALDH3A1 (Vasiliou et al. 1999). The expression of these genes is important in biotransformation and bioactivation of many xenobiotics and endogenous compounds.

Rat epidermal cells reproducibly exhibit a precipitous loss of *Cyp1a1* inducibility upon serial passages in culture with a consequent loss of sensitivity to toxicity from polycyclic aromatic hydrocarbons and heterocyclic amine substrates (Chun et al. 2000; Heimann and Rice 1983a; 1983b). This insensitivity becomes maximal in 50 generations (doublings) from primary culture (5 passages), indicating a physiological adaptation to the culture environment rather than a result of genomic aberrations that eventually occur concomitant with increasing colony forming ability. A similar loss of *Cyp1a1* inducibility can also be found in mouse skin tumors, where the physiological basis for such phenomena remains unknown (Reiners et al. 1998). In rat cells, the loss of *Cyp1a1* inducibility is not due to loss of AHR expression or dysfunction (as transfected DRE-luciferase constructs remain AHR-responsive), but a stepwise process that can be reversed upon inhibition of protein synthesis (Monk et al. 2003).

Stimulation of CYP1A1 inducibility by translational blockade is widely known as superinduction, in which cotreatment of cells with a protein synthesis inhibitor, such as cycloheximide (CHX), in the presence of TCDD or other AHR agonists markedly increases CYP1A1 expression compared to treatment with either the agonist or CHX alone (Lusska et al. 1992; Ma et al. 2000). In addition to CYP1A1, superinduction is also observed with other AHR target genes, such as ALDH3A1, CYP1A2, NMO1, and TiPARP (Joiakim et al. 2004; Ma 2002; Takimoto et al. 1991). Mechanistically, superinduction implicates the presence of a CHX-sensitive protein that negatively regulates AHR signaling, and inhibition of its synthesis contributes to prolonged AHR action (Ma and Baldwin 2000). This negative protein regulator may be agonist-inducible or short-lived and labile, and thus sensitive to inhibitors of protein synthesis. This unknown regulator was proposed to promote proteasomal degradation of ligand-activated AHR based on studies in the mouse hepatoma cell line (Hepa-1c1c7) where CHX stabilized AHR proteins in the nucleus, and inhibitors of the 26S proteosome led to superinduction of *Cyp1a1* (Ma and Baldwin 2000; 2002). In contrast to these previous findings in mice, in human breast epithelial cell line (MCF10A), CHX was reported to potentiate AHR loss, despite causing *CYP1A1* superinduction (Joiakim et al. 2004). The inconsistency between these two studies may arise from tissue- or species-specific variations, which could influence AhR dynamics differently. For instance, although AhR stability shows pronounced effects in some systems (Rudyak et al. 2023), other factors may play a more prominent role in different contexts.

Based on our previous observation that, after serial passages, rat epidermal cells lose *Cyp1a1* inducibility despite functional AHR signaling, we hypothesized that the diminishing gene induction was a result of epigenetic alterations. To test our hypothesis, we conducted a screening of 148 epigenetic modulators to identify candidates responsible for repressing TCDD-induced CYP1A1 expression in human and rat keratinocytes. In addition to identifying candidates that mimic CHX-mediated superinduction, our study also explored species-specific variations in the impact of epigenetic

modulators on CYP1A1 inducibility. Given the crucial role of CYP1A1 in procarcinogen activation (Androutsopoulos et al. 2009) and drug metabolism (Mescher and Haarmann-Stemmann 2018), our findings underscore the importance of epigenetic factors when extrapolating results from rodent models to humans or investigating clinical responses related to xenobiotic- or drug-drug interactions.

Materials and Methods

Chemicals. Cycloheximide (purity ≥ 90%) was purchased from Sigma-Aldrich. TCDD was from the National Cancer Institute (NCI) Chemical Carcinogen Repository. The epigenetics screening library and the individual epigenetic modulators were obtained from Cayman Chemical. The list of all library compounds can be found in the Supplementary Excel spreadsheet.

Cell Culture. Rat epidermal keratinocytes (passage > 20) were originally derived from minced tissues of adult Sprague-Dawley rats (Charles River Laboratories) (Heimann and Rice 1983b). Rat cells were chosen for this study due to their compatibility with the Rheinwald-Green culture system, which better preserves the physiological state of keratinocytes compared to the low calcium medium typically used for mouse epidermal cells (Allen-Hoffmann and Rheinwald 1984). This choice also ensures continuity with prior research. Both the rat epidermal keratinocytes and the human line of spontaneously immortalized epidermal keratinocytes (SIK) (Rice et al. 1993) were grown with 3T3 feeder layer support in a medium containing a 2:1 mixture of Dulbecco Vogt Eagle's and Ham's F-12 media supplemented with 5% fetal bovine serum, 0.4 µg/ml hydrocortisone, 5 µg/ml insulin, 5 µg/ml transferrin, 0.18 mM adenine, 10 ng/ml epidermal growth factor, and 10 µM Rho kinase inhibitor Y-27632 (Chapman et al. 2010). SIK cells (passages 20-25), which exhibit only a single chromosomal aberration and are nearly indistinguishable from normal human epidermal keratinocytes by multiple

criteria, including morphology and gene expression (Rea et al. 2006), show minimal deviation from normal, apart from elevated telomerase activity. Cells were grown at 37°C in 5% CO_2, and the medium was changed every two days. During treatments, the cells were incubated with the indicated chemicals in medium without epidermal growth factors and Y-27632. CHX was used at 30 µg/ml (or 100 µM) in medium on the basis of its concentration dependence in stimulating TCDD action (Supplementary Figure 1).

Quantitative RT-PCR. Cell samples were harvested in TRIzol reagent (Invitrogen), and RNA was isolated by phenol/chloroform extraction followed by ethanol precipitation. cDNA was synthesized with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and used for fast qPCR with TaqMan assays (Applied Biosystems). The qPCR reactions were run on the QuantStudio 3 Real-Time PCR System (Applied Biosystems). β-Glucuronidase (GUSB) was used as a reference for normalization as it is reported to be one of the most suitable endogenous control genes (Smith et al. 2020) and has proven to be reliable in lack of response to culture treatments in our lab over the years.

Ethoxyresorufin-O-deethylase (EROD) assay. Cells were seeded into a black-walled 96-well plate at 90% confluence and allowed to grow overnight before they were treated with 10 nM TCDD and 25 µM of the library chemicals. After 24-hr incubation, the cells were rinsed once with serum-free medium and incubated in this medium with 4 µM 7-ethoxyresorufin at 37°C. After 2 hr, the fluorescence intensity was measured using a SpectraMax iD3 Multi-Mode Microplate Reader (Molecular Devices) with excitation at 560 nm and emission at 600 nm. Readings from cells treated with 0.1% DMSO were used for background correction.

In-Cell Histone Deacetylase (HDAC) Activity Assay. Cells were seeded into a black-walled 96-well plate at 90% confluence and allowed to grow overnight before treatments. After 8-hr incubation with the indicated chemicals, HDAC activity was measured using a cell-permeable substrate following the

manufacturer's protocol (Cayman Chemical). Briefly, the cells were rinsed once with assay buffer and incubated with the HDAC substrate in serum-free medium at 37°C. After 2 hr, the cells were lysed by 15-minute incubation with lysis/developer solution. The fluorescence intensity was measured on the microplate reader with excitation at 350 nm and emission at 450 nm. Readings from culture medium were used for background correction.

Statistical Analysis.

Unpaired t-test was used to compare differences between 2 treatment groups. Comparisons between multiple groups were determined using ANOVA, with Dunnett's post-hoc test to calculate individual differences. All statistical analyses were performed on GraphPad Prism 10.

Results

Transcriptional effect of CHX on TCDD-elicited gene expression

Maximal *Cyp1a1* induction was observed by 6-8 hr treatment of rat keratinocytes with TCDD and protein synthesis inhibitors (Supplementary Figure 2). Hence, 8 hr was used as the incubation time for the following experiments. Since superinduction was previously observed with other AHR target genes, in addition to CYP1A1, the expressions of CYP1B1, ALDH3A1, and COX2 were also examined using real time qPCR.

When rat keratinocytes were treated with TCDD in the presence of CHX, *Cyp1a1* expression was induced by >47,000-fold compared to untreated rat cultures (Figure 1A). This superinduction generated levels of *Cyp1a1* mRNA significantly greater than that achieved by CHX or TCDD alone (47-fold and 79-fold respectively). Induction of the three other target genes was less remarkable. For example, in

Aldh3a1 expression, CHX alone had minimal impact, while TCDD alone increased its transcription by 15-fold, and the combination enhanced it by about 60-fold. *Cyp1b1* and *Cox2* mRNAs showed different responses: TCDD alone led to modest increases (3- and 2-fold respectively), CHX alone caused substantial increases (10- and 100-fold respectively), and the combined treatment resulted in only slightly higher levels (30- and 300-fold respectively) than with CHX alone. The pronounced effect of CHX alone on *Cox2* could be explained by its translational blockade of IκBα synthesis (a negative regulator of NF-κB) and hence, stimulation of downstream *Cox2* expression (Newton et al. 1997).

Superinduction of *CYP1A1* in human keratinocytes was significant but notably lower than that in rat epidermal cells. The combined treatment of TCDD and CHX produced a 500-fold induction in *CYP1A1*, and the level achieved by CHX or TCDD alone was 15- and 30-fold, respectively. *CYP1B1* induction was substantial but smaller, where the combined treatment produced a 300-fold induction while CHX and TCDD produced 10- and 20-fold induction, respectively. *ALDH3A1* showed minimal induction (< 2-fold) by single or combined treatments. For *COX2*, similarly to the rat, CHX was the primary inducer (25-fold) not TCDD (< 2-fold), and the combination gave about 50-fold induction.

Enhancement of TCDD-elicited EROD activity by epigenetic modulators

Previous studies in mice suggest that inhibition of proteasomal degradation was involved in *Cyp1a1* superinduction (Ma and Baldwin 2000; 2002). However, our initial efforts with inhibitors targeting a variety of protease activities were ineffective in altering the magnitude of *Cyp1a1* superinduction (Supplementary Table S1). To test our hypothesis that the labile protein could have a role in epigenetic regulation, we screened 148 epigenetic modulators to identify candidates that mimic CHX and enhance TCDD-induced CYP1A1 expression in human and rat cells.

EROD activity was used to measure CYP1A1 induction in cells cotreated with TCDD and the 148 known epigenetic modulators. TCDD alone induced a 40-fold increase in EROD activity compared to the control. Since CHX inhibits protein synthesis, including the newly synthesized CYP1A1 protein, the combined treatment of TCDD plus CHX reduced the EROD activity to 2-fold compared to control (Supplementary Figure S3). In the first screening, 10, 25, and 50 μM of library chemicals were tested in rat keratinocytes for 24 h. Nearly 25% of these chemicals, at the concentration of 50 μM, resulted in toxicity and cell death. At 10 and 25 μM, 16% and 22% of the library chemicals increased EROD activity by at least 2-fold, respectively. Therefore, the concentration of 25 μM was selected for the following experiments. When compared to cells treated with only TCDD, 32 library chemicals were found to significantly enhance EROD activity by at least 2-fold in rat keratinocytes (Figure 2). Among the 32 library chemicals, the top 10 modulators were exclusively HDAC inhibitors, and the rest included inhibitors of protein arginine methyltransferase (PRMT), lysine-specific demethylase (LSD), and bromodomain (BRD) (Figure 2B). In human cells, however, only HPOB, a selective inhibitor of HDAC6 (Lee et al. 2013), was positive, producing a 2-fold enhancement in EROD activity. Details of the screening results and statistical comparisons can be found in the Supplementary Excel spreadsheet.

Effects of HDAC inhibitors and SIRT activator on TCDD-induced gene expression

Based on functional and sequence homology, mammalian HDAC proteins are divided into classes I, II, III, and IV (Seto and Yoshida 2014). Of the 32 library chemicals that increased TCDD-induced EROD activity in rat cells, 4 modulators with different HDAC targets were examined for their transcriptional effects on AHR target genes. These 4 modulators include CXD101, a selective inhibitor for class I HDACs (Eyre et al. 2019); Nexturastat A, an inhibitor specifically against HDAC6 (a class II HDAC) (Bergman et al. 2012); SAHA (also known as Vorinostat), a broad-spectrum inhibitor targeting class I and II HDACs (Marks and Breslow 2007); and BML-278, an activator of SIRTs (the class III HDACs)

(Mai et al. 2009). As shown in Figure 3A, TCDD, in combination with CXD101, led to a 1,500-fold *Cyp1a1* induction in rat vs. 300-fold in human keratinocytes, and with Nexturastat A, the induction was 4,700-fold in rat vs. 200-fold in human cells. However, none of the 4 chemicals stimulated *Cyp1b1* inducibility in rat cells (except marginally CXD101) (Figure 3B). In human cells, CXD101 and BML-278 increased TCDD-induced *CYP1B1* expression, whereas Nexturastat A and SAHA decreased the expression. The expression pattern of rat *Aldh3a1* was similar to that of *Cyp1a1* (with CXD101 and Nexturastat A enhancing the expression and BML-278 and SAHA producing no significant changes), suggesting these two genes could have common epigenetic regulation (Figure 3C). The levels of human *ALDH3A1* transcripts were not further increased by any of the combinations. Finally, in both species, COX2 gene was weakly induced by TCDD (~ 2-fold) (Figure 3D). Yet, in the presence of Nexturastat A, *Cox2* expression was enhanced in rat keratinocytes. By contrast, Nexturastat A (and SAHA) generated the opposite effects in human keratinocytes, where the two chemicals significantly decreased the expression.

Decreased intracellular HDAC activity by CHX and the 4 library chemicals

Inhibition of HDACs seemed to stimulate TCDD-elicited CYP1A1 expression. The estimated half-life of HDAC1 and 2 is around 24 hr (Jamaladdin et al. 2014), and it is likely that CHX could stimulate CYP1A1 inducibility through blockade of HDAC synthesis. As shown in Figure 4, after 8-hr incubation, the cellular HDAC activity was decreased to 70~75% by CHX, a level comparable to those by Nexturastat A or BML-278. When incubated with SAHA, the enzyme activity was further decreased to 50~60%, and CXD101 demonstrated the strongest inhibition by decreasing the HDAC activity to 25~30%. Cotreatment with TCDD produced no significant changes.

The degree of HDAC inhibition, however, did not correlate strictly with the level of CYP1A1 induction. For example, among the 4 library chemicals, CXD101 produced the strongest HDAC inhibition.

However, in the qPCR experiment, it stimulated TCDD-induced CYP1A1 expression to the level comparable to that by Nexturastat A. This could be due to target specificity since this HDAC assay does not distinguish among different classes, and CXD101 acts on multiple class I HDACs, while Nexturastat A is more selective against HDAC6. Finally, although in both species, CHX and the 4 library chemicals are equally effective at inhibiting HDAC activity, they all produced a more pronounced impact on TCDD-induced CYP1A1 expression in rat than in human cells (Figure 3A), indicating that the rat *Cyp1a1* gene is more sensitive to histone modifications in the culture environment.

Discussion

Individual genes respond to xenobiotic stimulation in different ways due to the intricate variations in their genetic makeup, regulatory elements, and epigenetic machinery. The epigenome plays a pivotal role in shaping the interplay between transcription factors and chromatin landscape, determining the accessibility and inducibility of a gene (Lim et al. 2013). This delicate process is governed by epigenetic enzymes as they constantly add or remove chemical signatures on DNA and histone proteins, marking a gene active or inactive (Allis and Jenuwein 2016). Given the substantial disparity between the microenvironment in culture and *in vivo*, cultured cells frequently display properties that deviate from their native state. This phenomenon has gained significant attention, particularly in stem cell culture, where efforts are focused on developing conditions to enable their clinical use. While considerable emphasis has been placed on DNA methylation (Rebuzzini et al. 2016), recent studies highlight the influence of cellular metabolites on other chromatin modifications as well (Dai et al. 2020; Harvey et al. 2019). These changes may manifest over time and exhibit species-specific differences. For example, primary culture of mammalian hepatocytes exemplifies rapid responses, where significant chromatin alterations and shifts in transcription factor activities occur within hours in culture, thereby limiting their

utility in toxicity testing (Levy et al. 2015; Seirup et al. 2022). Likewise, in our study, despite similar patterns of superinduction in both rat and human epidermal cells, differences in sensitivity between the two species could reflect the variations in cellular metabolites or stabilities of components within the histone deacetylation pathway.

Histone acetylation is controlled by histone acetyltransferase (HAT) and HDAC, where HATs catalyze the transfer of an acetyl group to the lysine residues in proteins, and HDACs remove it (Eckschlager et al. 2017). Acetylation of histones has been associated with active transcription due to the weakened histone-DNA interactions and a more open chromatin structure, whereas HDACs are commonly recruited to active genes for transcriptional repression (Shahbazian and Grunstein 2007). For example, recruitment of HDAC1 by Elk-1 was shown to repress the expression of its downstream gene c-*fos*, and inhibition of HDAC was found to prevent the repression and even enhance the basal level of c-*fos* expression (Yang et al. 2001). Similarly, by increasing acetylation of histone H3 and H4 at the promoter region, HDAC inhibitors have been consistently reported to activate the expression of cyclin-dependent kinase inhibitor p21(cip1/waf1) (Ocker and Schneider-Stock 2007).

In this study, HDAC inhibitors (CXD101 and Nexturastat A, were found to synergize with TCDD to elevate CYP1A1 transcript levels (Figure 3A), in line with previous studies that histone acetylation plays a role in transcriptional activation of CYP1A1 gene (Garrison et al. 2000; Schnekenburger et al. 2007; Vorrink et al. 2014). The marginal effect of SAHA suggests that the concentration used may not have been optimal. In future studies investigating the effects of epigenetic modulators on superinduction, optimizing the concentration as the initial step would be advantageous.

Species-specific responses to TCDD and the HDAC inhibitors were observed, with human *CYP1B1* gene being more inducible than rat, and rat *Aldh3a1* gene being more sensitive than human (Figure 3B and C). In previous studies using cultured human cells, the HDAC inhibitors sodium butyrate and

trichostatin A were reported to increase basal and inducible *CYP1B1* transcription in a cell type-specific manner (Nakajima et al. 2003; Park et al. 2015). Increased expression of *ALDH3A1* has been associated with chemoresistance and is epigenetically regulated by histone demethylation in human cancer cells (McLean et al. 2023). Although the epigenetic control of rat *Aldh3a1* remains relatively unexplored, our findings suggest a regulatory mechanism akin to that observed in *Cyp1a1* as underscored by their similar transcriptional responses to the library chemicals. The rat *Cox2* gene showed limited responsiveness to the 4 library chemicals, except for Nexturastat A. Notably, in the presence of TCDD, Nexturastat A significantly induced the expression of *Cox2* in rat cells. In human cells, however, Nexturastat A and SAHA were found to inhibit *COX2* expression, with the inhibitory effect further strengthened in the presence of TCDD. This response in human cells is consistent with earlier reports indicating that HDAC inhibitors can suppress *COX2* induction in various human cell lines, potentially through AP-1 inhibition (Subbaramaiah et al. 1998; Tong et al. 2004). This aligns with the synergistic effects observed in the combined treatments with TCDD, as TCDD has also been reported to inhibit AP-1 activity (Suh et al. 2002). Finally, in human but not rat cells, BML-278, increased basal levels of *CYP1A1*, *CYP1B1*, and *ALDH3A1* transcripts, in line with a prior study demonstrating that SIRT1 is essential for basal AHR activity in keratinocytes (Ming et al. 2015).

In human cells, although CXD101 and Nexturastat A enhanced TCDD-induced CYP1A1 expression, the elevated mRNA levels do not contribute to increased EROD activity (Figure 2B). Unlike the rat cells, the human CYP1A1 activity in the cotreatment groups did not differ significantly from that achieved by TCDD alone. BML-278 and SAHA even decreased EROD activity. This inconsistency between mRNA transcript level and the ultimate protein product can be attributed to various factors, including mRNA stabilization, translational efficiency, or protein turnover rate. Because HDAC inhibitors are known to target other cytoplasmic proteins and act beyond their typical role in chromatin remodeling and gene

expression (Choudhary et al. 2009; Schmitz and de la Vega 2015), their broad impacts could therefore lead to the observed inconsistency.

The observed reduction in HDAC activity by CHX (Figure 4) implies a possible mechanism wherein this decrease in enzyme activity mimics the effect of HDAC inhibitors, contributing to the phenomenon of superinduction. However, instead of a single regulatory event, superinduction likely results from several distinct processes, including increased mRNA stabilization, loss of labile transcriptional repressor, and disruption of the negative feedback loops due to inhibited translation (Mahadevan and Edwards 1991; Radulovic and Tronson 2008). Our study indicates that loss of epigenetic regulation, particularly HDAC activity, by protein synthesis inhibition could also contribute to CYP1A1 superinduction, introducing an additional factor to the phenomenon. Although CHX and the four library chemicals were equally effective at inhibiting HDAC activity in both species, they produced a more pronounced increase in TCDD-induced CYP1A1 expression in rat cells than in human cells (Figures 1 and 3). This suggests that the rat *Cyp1a1* is more sensitive to histone modifications than the human *CYP1A1*, thereby exhibiting a greater degree of superinduction.

CYP1A1 stands out as a significant P450 enzyme responsible for metabolic activation of environmental procarcinogens (Androutsopoulos et al. 2009). Loss of its activity in rodent models can pose challenges in extrapolating carcinogenicity test results (Reiners et al. 1998). Additionally, understanding the impact of chemicals and drugs on CYP1A1 inducibility is crucial for predicting the toxicokinetic of chemical action in *vivo* (Mescher and Haarmann-Stemmann 2018). In this study, we demonstrated that inhibition of protein synthesis and HDAC activity significantly enhances TCDD-induced CYP1A1 expression in both rat and human keratinocytes, with a more pronounced effect in rat cells. This suggests that the rat *Cyp1a1* gene is more responsive to histone modifications than the human CYP1A1. Our results also revealed species-specific differences in the regulation of other AHR target genes, such as CYP1B1,

ALDH3A1, and COX2. These findings provide valuable insights into the epigenetic regulation of CYP1A1 and indicate that differences in HDAC activity and histone sensitivity may contribute to species-specific responses to xenobiotics. Future studies on the epigenetic regulation of CYP1A1, including in vivo, can prioritize targets of those effective library modulators, for example class I HDACs or HDAC6. Finally, this study could also lay the groundwork for a more in-depth exploration of superinduction by comparing the epigenomes of cells treated with or without protein synthesis inhibitors.

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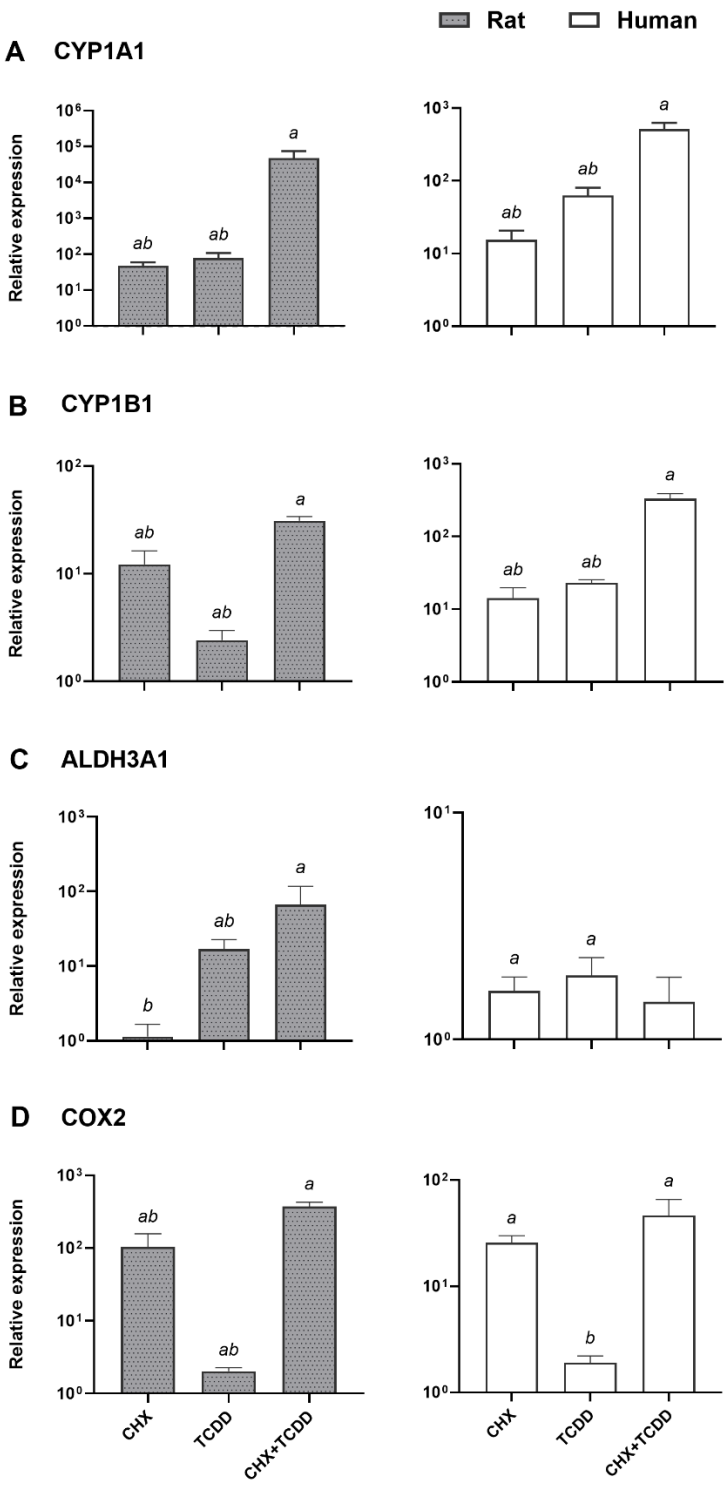


Figure 1. Effects of CHX on TCDD-elicited gene expression. Rat and human keratinocytes were harvested after 8-hr incubation with indicated chemicals (TCDD: 10 nM, CHX: 100 μM). The mRNA levels of target genes are expressed as relative to untreated control (10^0 in the figure). Results are presented as the mean ± SD from three independent experiments. *a* indicates significant difference from untreated control, and *b* indicates significant difference from the CHX+TCDD group ($p < 0.05$).

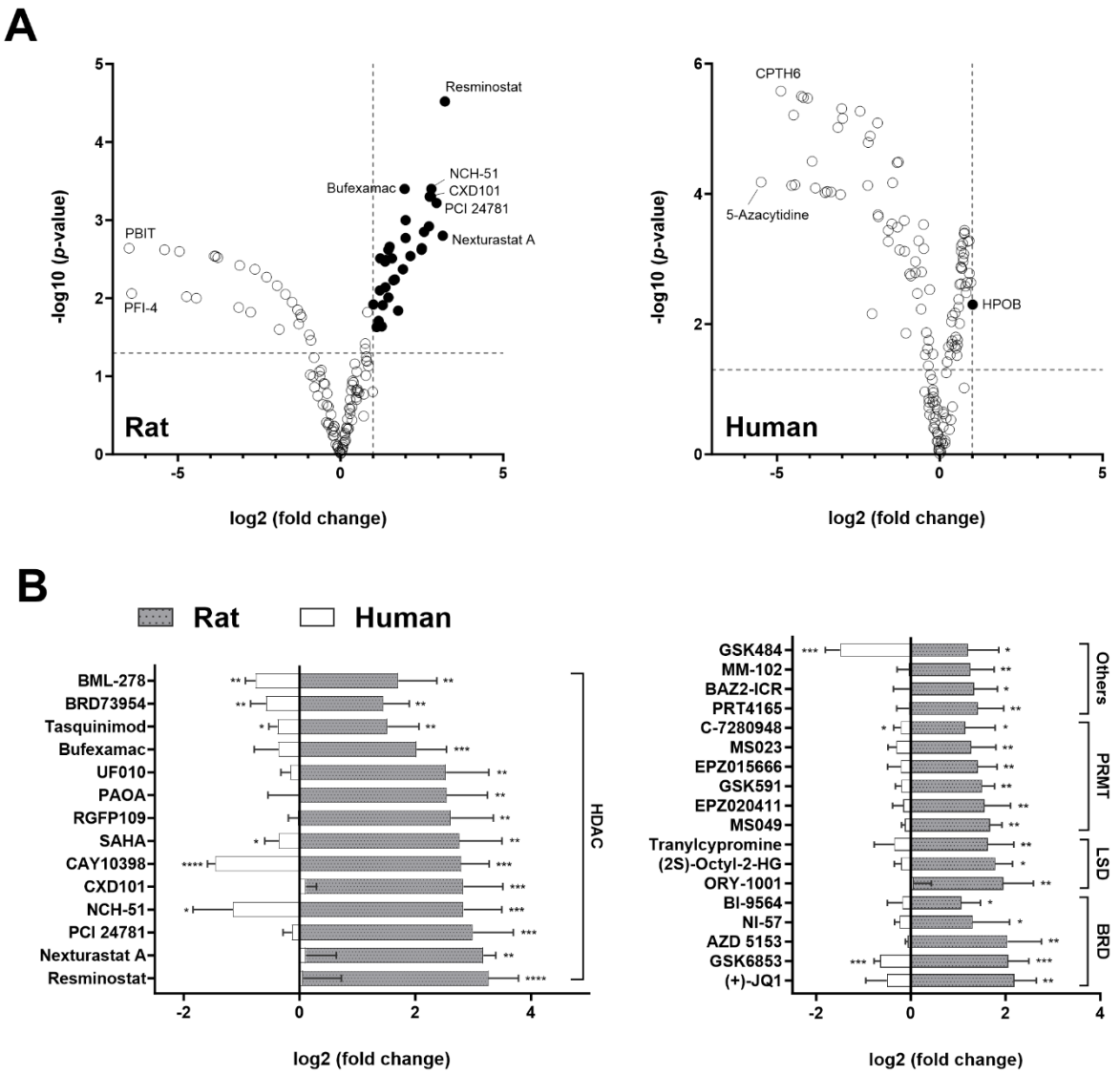


Figure 2. Changes of TCDD-induced EROD activity by epigenetic modulators. Rat and human keratinocytes were assayed for EROD activity after 24-hr incubation with 10 nM TCDD and 25 μM library chemicals. In both rat and human cells, TCDD alone generated about 40-fold higher induction than the DMSO control. Preliminary experiments with 8-hr incubation in rat cells showed no significant changes in EROD activity by any of the library chemicals. Cells treated with 0.1% DMSO were used for background correction, and the EROD activity is presented as log2 fold change between cotreatment groups and TCDD-only group. The results were obtained from three independent experiments. (A) Volcano plots showing groups with ≥ 2-fold enhancement (black dots). The vertical dashed line indicates the 2-fold increase, and the horizontal dashed line indicates statistical significance with a *p*-value of 0.05. (B) Bar charts showing the 32 library chemicals, their molecular targets, and their effects on TCDD-induced EROD activity. Data are presented as the mean ± SD. Significance differences from TCDD-only group are indicated (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

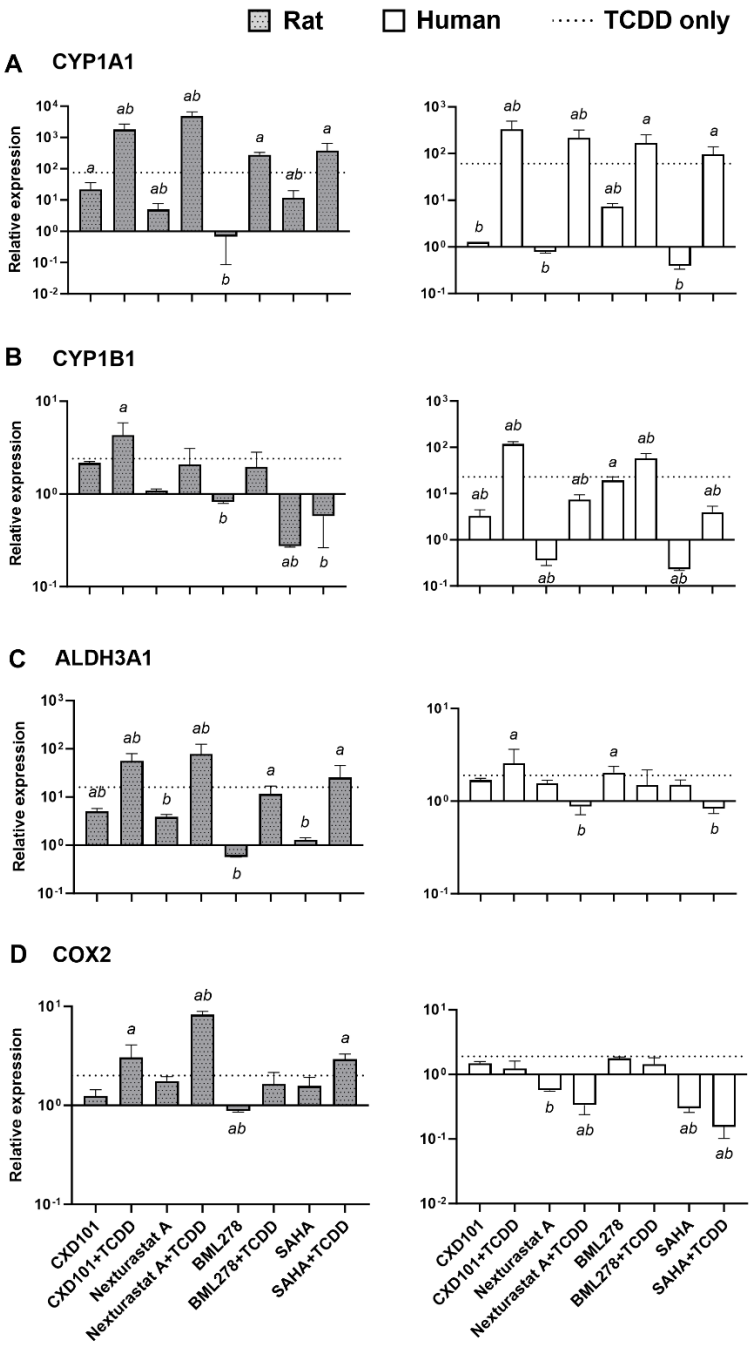


Figure 3. Effects of CXD101, Nexturastat A, BML-278, and SAHA on TCDD-elicited gene expressions. Rat and human keratinocytes were harvested after 8-hr incubation with 25 μM indicated chemicals ± 10 nM TCDD. The mRNA levels of (A) CYP1A1, (B) CYP1B1, (C) ALDH3A1, and (D) COX2 are expressed as relative to untreated control (10^0 in the figure). Results are presented as the mean ± SD from three independent experiments. *a* indicates significant difference from untreated control, and *b* indicates significant difference from TCDD-only group ($p < 0.05$).

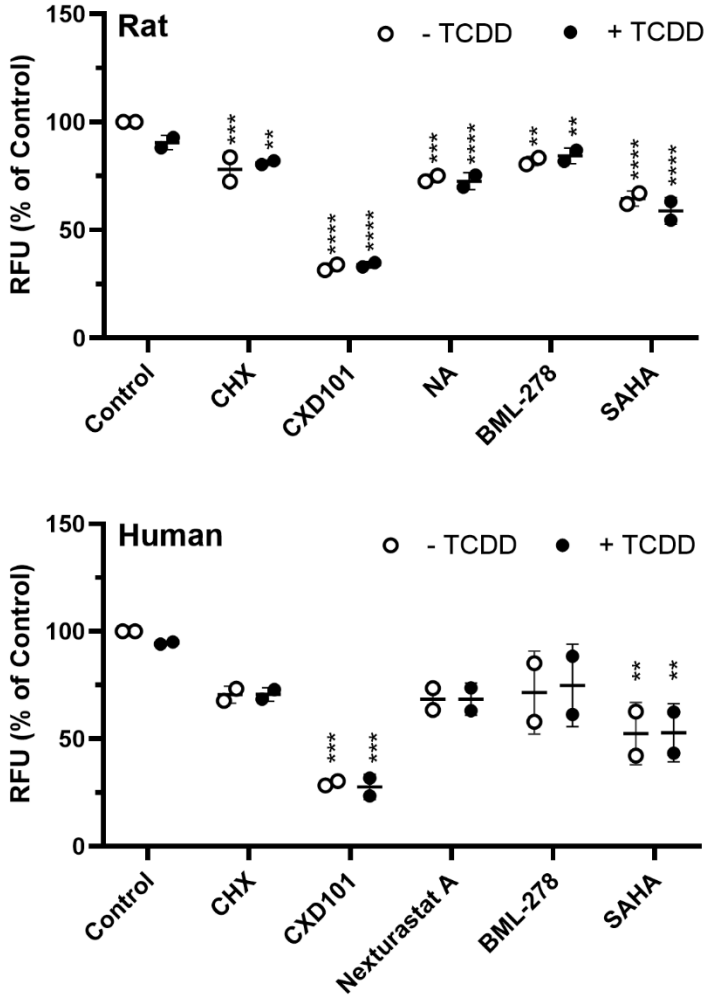
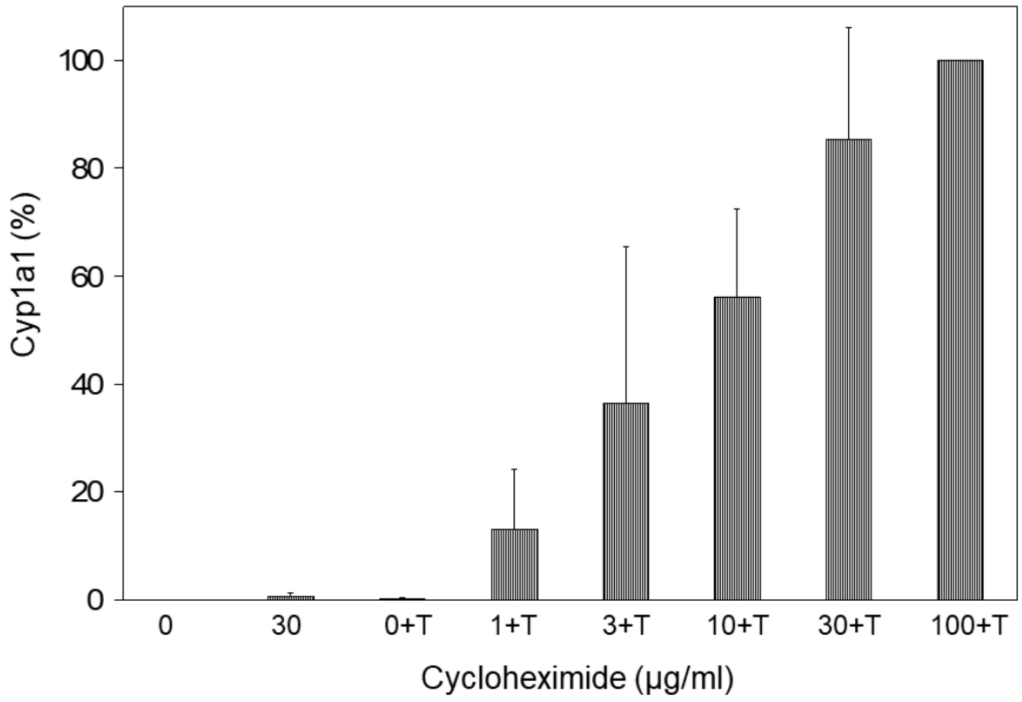
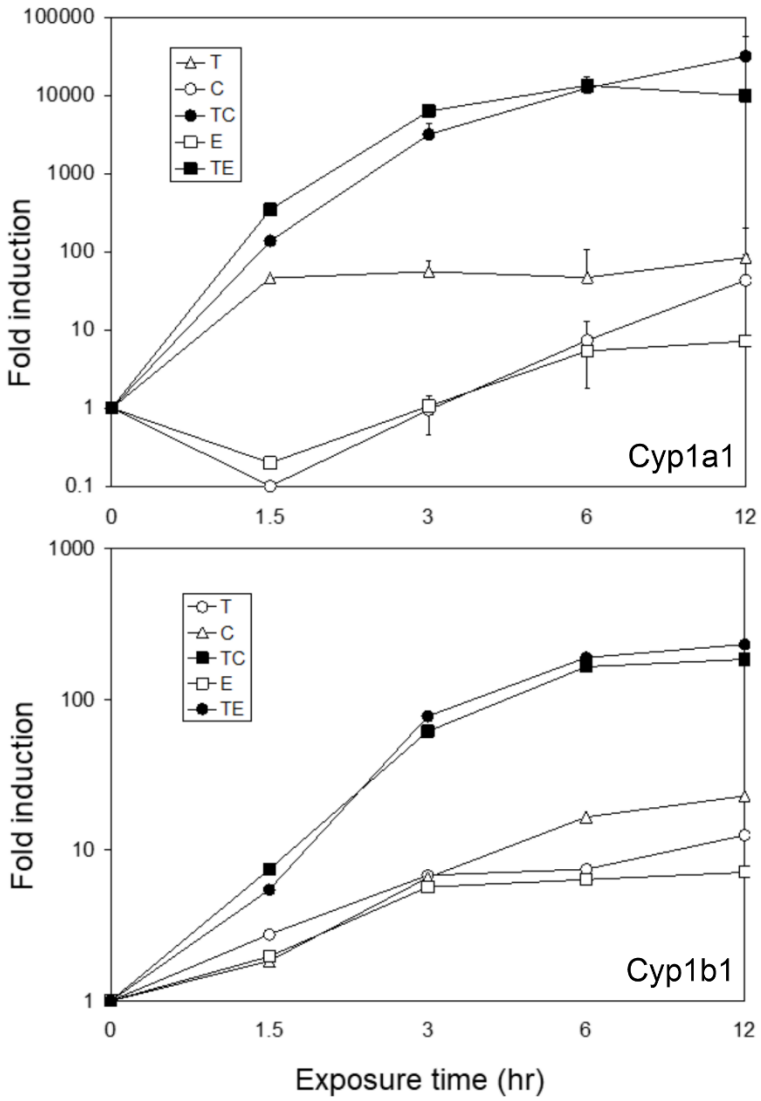


Figure 4. Reduced HDAC activity in cells treated with CHX and the library chemicals. Rat and human keratinocytes were assayed for HDAC activity after 8-hr incubation with 100 µM CHX or 25 µM library chemicals ± 10 nM TCDD. Values from culture medium were used for background correction and the HDAC activity is presented as % of control (cells treated with 0.1% DMSO). Results are presented as the mean ± SD from two independent experiments. Significant differences from control are indicated (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$). Please note that Nexturastat was used in place of HPOB in Figures 3 and 4 because the former is more potent (IC50 of 5 nM vs 56 nM for the latter).



Supplementary Figure S1. *Cyp1a1* mRNA level as a function of cycloheximide concentration. Newly confluent epidermal cultures from rat keratinocytes were treated with cycloheximide at the indicated concentrations. Treatment in addition with TCDD (10 nM) is indicated by + T. Values were normalized to the level of reference gene *Gusb*. Figure shows the means and standard deviations from three experiments, where the value for 100 + T was set as 100%.



Supplementary Figure S2. Time course of *Cyp1a1* and *Cyp1b1* induction. Parallel cultures from rat keratinocytes were treated with 10 nM TCDD (T), cycloheximide (C), emetine (E) or TCDD plus protein synthesis inhibitor as indicated. At intervals, cultures were harvested in Trizol and mRNA levels were measured by real time PCR. Values of *Cyp1a1* and *Cyp1b1* expressions were first normalized to the mRNA level of the housekeeping gene *Actb* and then to give fold induction over parallel untreated cultures. The curve shows the average of two independent experiments for TCDD + cycloheximide and one experiment with emetine.

Supplementary Table S1. Effects of protease inhibitors on *Cyp1a1* induction*.

Inhibitor	Concentration (μM)	% Cyp1a1 Induction	Note
AEBSF	30	94 ± 9	Inhibitor of serine proteases
PMSF	1000	85 ± 31	Inhibitor of serine proteases
MG132	10	76 ± 27	Inhibitor of 26S proteasome and calpain
	12	148 ± 54	
	15	81 ± 8	
	30	133 ± 35	
ALLN	25	119 ± 15	Inhibitor of cysteine proteases
AALVS	10	114 ± 9	Inhibitor of proteasome
Bortezomib	0.05	161 ± 34	Inhibitor of 20S proteasome
	0.2	150 ± 2	
	2.5	52 ± 27	
	5	150 ± 96	
	10	208 ± 179	
Phenanthroline	100	53 ± 4	Inhibitor of metalloproteases

[*] % induction relative to TCDD + CHX (set to 100%) in rat keratinocytes. Results are shown as mean ± SD from three experiments. None of the cultures were altered in morphology during treatment. AEBS and 1,10-phenanthroline were purchased from Sigma; PMSF from Research Organics; MG132, ALLN, and AALVS were obtained from Biomol; and bortezomib was from LC Labs.