A comprehensive evaluation of the effects of RNA-editing proteins ADAR and ADARB1 on the expression of the drug-metabolizing enzymes in HepaRG cells

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Running Title: Regulation of the drug-metabolizing enzymes by the ADARs

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Abbreviations: Transcription Factor (TF), knockdown (KD), 3-Methylcholantheine (3MC), cytochrome P450 (CYP), UDP glucuronosyltransferase (UGT), the adenosine deaminase acting on RNA (ADAR).
Abstract

Two RNA editing proteins, the adenosine deaminase acting on RNA, ADAR, and ADARB1, broadly regulate gene expression in editing-dependent and editing-independent manners. Previous studies showed that the expression of the drug-metabolizing cytochrome P450s (CYPs) and UDP glucuronosyltransferases (UGTs) changes upon knock-down (KD) of ADAR or ADARB1 in different hepatic cell lines. To systematically survey the effects of these two ADARs on the expression of CYPs and UGTs, we used siRNA in HepaRG cells and tested the association between the expression of the CYPs and ADARs in a liver sample cohort (n=246). KD of ADAR increased the expression of the CYP3As and CYP2C9 and reduced the expression of the others, while KD of ADARB1 reduced the expression of nearly all genes tested. ADAR KD also suppressed the induction of most CYPs, while ADARB1 KD had mixed effects depending on the inducer/gene combination. CYP expression was positively associated with both ADARs in liver samples, consistent with the KD results. However, after adjusting for the expression of transcription factors (TFs) known to regulate CYP expression, the associations disappeared, indicating that the effects of ADAR or ADARB1 primarily occur through TFs. Moreover, we found that the expression of normally-spliced CYP3A5 transcripts is increased in both KDs, indicating a direct effect of the ADARs on promoting the usage of the cryptic splice site generated by CYP3A5*3. Taken together, our results revealed the non-overlapping regulatory effects of ADAR and ADARB1 and supported their broad roles in controlling the expression of drug-metabolizing enzymes in the liver.
Significance statement

Here we systematically surveyed the roles of ADAR and ADARB1 in both basal and induced expression of drug-metabolizing enzymes and assessed their co-expression in liver samples. Our results support that ADAR and ADARB1 regulate the expression of the drug-metabolizing enzymes in the liver, suggesting that factors affecting ADAR expression also have the potential to impact drug metabolism.

Introduction

Inter-person variability in drug metabolism significantly affects drug exposure and treatment outcomes. Genetic biomarkers in drug-metabolizing enzymes have been pivotal in guiding personalized drug therapy. Most currently clinically-actionable pharmacogenetic biomarkers disrupt the protein-coding sequence or canonical splicing sites leading to reduced enzyme activity or no functional proteins. However, factors that account for the large variation in the expression of drug-metabolizing enzymes between individuals remain largely unknown (Roden et al., 2019). Incorporating the variation arising from the many interwoven layers controlling the expression of drug-metabolizing enzymes will likely improve the predictive power of current genetic biomarkers (Sadee et al., 2023).

The road from gene to protein is complex (see review by Buccitelli and Selbach, 2020), with numerous phases determining protein abundance and activity, including post-transcriptional modification. The adenosine deaminase acting on RNA (ADAR) proteins are RNA editing enzymes that post-transcriptionally modify RNA (Shevchenko and Morris, 2018). There are two catalytically-active ADAR family members in humans, ADAR1 (gene symbol ADAR) with two alternate isoforms, ADAR1p110 and
ADAR1p150, and ADAR2 (gene symbol ADARB1), and both have non-overlapping targets (Tan et al., 2017). To be consistent with standard nomenclature, we use ADAR and ADARB1 to refer to the gene, mRNA, and protein for these two ADARs. Canonic ally, the ADARs bind to double-stranded RNA (dsRNA) and edit adenosines (A) to inosines (I) (commonly referred to as A-to-I editing) (Wang et al., 2017). This ADAR-mediated editing occurs in most human genes, primarily at Alu elements, because Alus promote the formation of dsRNA (Bazak et al., 2014). The editing of mRNA has clear implications for changing protein-coding sequences, but this appears to be relatively infrequent (Shevchenko and Morris, 2018). Instead, ADARs are more broadly implicated in the control of gene expression through numerous avenues, such as by altering RNA splicing (Solomon et al., 2013) and stability (Shevchenko and Morris, 2018), miRNA expression/processing and recognition of their target sequences (Cho et al., 2017), the binding/activity of other RNA-binding proteins (Licht and Jantsch, 2017), and these effects can occur independently of the A-to-I editing activity (Licht and Jantsch, 2017).

Recently, the Nakajima et al. group suggested the involvement of ADARs in the regulation of the expression of drug-metabolizing enzymes. Using siRNA-mediated ADARs knockdown (KD) experiments, they found that the expression levels of several cytochrome P450s (CYPs) changed, and these effects were likely mediated through regulation of liver enriched transcription factors (TFs) like the Aryl Hydrocarbon Receptor (AHR), Constitutive Androstane Receptor (CAR or NR1I3), Pregnane X Receptor (PXR or NR1I2), and Hepatocyte Nuclear Factor 4 Alpha (HNF4A) (Nakano et al., 2016; Nakano et al., 2019; Nozaki et al., 2019; Takemoto et al., 2021). These
results imply that the ADARs are involved in drug metabolism and pharmacokinetics (Nakano and Nakajima, 2018) and warrant further investigation.

These previous reports primarily focused on ADAR regulation of individual TF-CYP interactions. However, the CYPs are co-regulated by several TFs (Zanger and Schwab, 2013), and the results were generated in different hepatoma cell lines, which vary in the expression of the drug-metabolizing enzymes (Guo et al., 2011). To assess the degree to which ADARs control the expression of drug-metabolizing enzymes and to compare the effects between the two ADARs systematically, we used siRNAs to KD ADAR or ADARB1 in HepaRG cells and tested the changes in the expression of all drug-metabolizing CYPs, liver-expressed UDP glucuronosyltransferases (UGTs) and TFs that regulate their expression. We also tested the changes in the inducibility of drug-metabolizing enzymes. Finally, we tested the relationship between the expression of both ADARs, the TFs, and the drug-metabolizing enzymes in a liver sample cohort (n=246). Our results support the broad roles of ADAR and ADARB1 in regulating the expression of the drug-metabolizing enzymes. Our results also suggest that ADAR and ADARB1 have distinct roles in controlling the expression of the CYPs and UGTs.

Materials and Methods

Liver samples and IRB

The Cooperative Human Tissue Network (CHTN, Bethesda, MD) provided the human liver samples, demographic information, and sample processing steps were published (Collins and Wang, 2021). The University of Florida Institutional Review Board (IRB) approved the study.
Cell culture, transfection, and inducer treatment:

HepaRG® cells were purchased from Biopredic International (Saint Grégoire, France) and cultured and differentiated according to the manufacturer's protocols. Undifferentiated cells were maintained in HepaRG growth media: William's E Media (A1217601, Gibco™, ThermoFisher), HepaRG Growth media supplement (ADD711C, Biopredic International), 1x GlutaMAX™ (Gibco™, ThermoFisher), 1x penicillin/streptomycin (Gibco™, ThermoFisher). Cell differentiation and maintenance occurred in the same media, but the HepaRG differentiation supplement (ADD721C, Biopredic International) was substituted for the growth supplement. Undifferentiated cells were seeded into 12-well plates, expanded, and differentiated. Differentiated HepaRG cells were transfected using Lipofectamine™ RNAiMAX (ThermoFisher) following the manufacturer's protocol with 20 nM Silencer™ Select siRNAs (ThermoFisher): ADAR (siRNA ID#: s1007), ADARB1 (siRNA ID#: s1010), or the Silencer™ Select negative control #1. Twenty-four hours after transfection, the media was replaced with HepaRG differentiation media. Forty-eight hours post-transfection, the media was changed to a serum-free induction media composed of William's E Media (A1217601), 1x GlutaMAX™, 1x penicillin/streptomycin, 1x ITS (41400045), 15 mM HEPES (Gibco™, ThermoFisher) with DMSO or different inducers. The final inducer concentrations were: rifampicin (10 µM), CITCO (5 µM), and 3MC (2.5 µM).

RNA extraction, cDNA synthesis, and qPCR:

RNA was extracted from HepaRG cells using TRI Reagent® (Zymo Research, CA, USA) and purified using the Direct-zol RNA Miniprep kit (Zymo Research, CA, USA). Due to supply chain issues, we used two kits to synthesize cDNA from 250 ng of...
RNA. The qScript XLT cDNA SuperMix (VWR, PA, USA) was used for ADAR, ADARB1, CYP3A4, CYP1A2, CYP2B6, and CYP2C9. For all other genes tested, we used the qScript Ultra Flex kit (VWR, PA, USA). Relative expression was obtained in both cases by comparing it to β-actin synthesized with the same cDNA kit. qPCR measurements were conducted on a Quantabio Q real-time PCR instrument (VWR, PA, USA). Most genes were measured using the SYBR green method with PerfeCTa SYBR® Green FastMix (VWR, PA, USA), except for CYP3A5, CYP2C9, and CYP2C19, which were measured using TaqMan assays with PerfeCTa FastMix II (VWR, PA, USA). Primers used for qPCR are listed in Supplemental Table 1.

Liver expression of the CYPs and TFs are published, and measurements of ADAR and ADARB1 via SYBR green followed the same procedure as previously reported (Collins and Wang, 2021).

Protein extraction and quantification

Protein from transfected HepaRG cells was extracted using ice-cold 1x RIPA Lysis Buffer (EMD Millipore) containing protease inhibitors (cOmplete™ Tablets, Roche). Total protein was quantified using the Bradford assay (ThermoFisher, CA, USA). Using the manufacturer's protocol, protein expression was measured via the capillary western blot Jess system (ProteinSimple, CA, USA). Briefly, protein lysates were diluted to 1 µg/ul using 0.1x sample buffer, and 2 µg protein was combined with the 5x fluorescent master mix, and heated at 95°C for 5 min. Primary antibodies used were: mouse anti-CYP3A4 (R&D MAB 9079, 1:20 dilution), mouse anti-β-actin (R&D MAB8929, 1:20 dilution), mouse anti-ADAR (SantaCruz SC-73408, 1:5 dilution), rabbit anti-ADARB1 (Proteintech 22248-1-AP, 1:10 dilution). Anti-mouse-HRP, Anti-rabbit-
HRP, and Anti-mouse-NIR were all used at 1:20 dilution (ProteinSimple, San Jose, CA, USA). Loading, separation, detection, and quantification were all conducted within the automated Jess system. β-actin was used to control for differences in protein loading and acted as an internal control for protein normalization of CYP3A4.

Data analysis

Analysis of the HepaRG qPCR data was conducted in R version 4.2.3, with the following packages: tidyverse v1.3.2 (Wickham et al., 2019), ggpubr v0.5 (Kassambara, 2023), gridExtra v2.3 (Auguié, 2017), data.table v1.14.6 (Dowle and Srinivasan, 2023), and ReadqPCR/NormqPCR v1.43.0 (Perkins et al., 2012). The Student’s t-test was performed in base R to compare dCT and normalized protein expression values. Two-way ANOVA was calculated using base R and estimated marginal means and 95% confidence intervals were computed using modelbased v0.8.6 [6]. Pearson correlation was used to determine the correlation between the expression of the ADAR family members in base R.

Association analysis:

A multiple linear regression model was used for testing the association between the expression of CYPs and ADAR, ADARB1 and TFs using Minitab 21 software. We used forward and backward stepwise regression to select the best predictors in the multiple linear regression models with a cutoff p-value of ≤0.05. Reported age, sex, and race were included as covariates in all models.
Results

The effects of siRNA-mediated KD of ADAR or ADARB1 on the expression of CYPs, UGTs, and TFs in HepaRG cells

We used siRNA to KD ADAR (targeting ADARp110 and p150) or ADARB1 in differentiated HepaRG cells. Expression of both genes was significantly reduced at the mRNA level (ADAR >80% reduction in siADAR; ADARB1 >70% reduction in siADARB1), and KD of ADAR did not impact ADARB1, or vice-versa (Supplemental Figure 1a). Protein levels of both ADAR family members were also reduced in the KDs (Supplemental Figure 2). We then measured the changes in gene expression in these cells, specifically: the phase I drug metabolizing enzymes (12 CYPs and CES1/2), eight liver-expressed phase II enzymes (the UGTs) (Tourancheau et al., 2018), and eight TFs known to be involved in the regulation of the CYPs and UGTs (Collins and Wang, 2021).

KD of ADAR significantly increased the expression of CYP2C9 and all CYP3As except for CYP3A5, decreased the expression of CYP2C8, CYP2B6, CYP2E1, CYP1B1, and CES2, and had no effects on the expression of CYP2C19, CYP2A6, and CES1 (Figure 1a and Supplemental Table 2). In contrast, of the UGTs tested, only UGT1A9 significantly decreased in siADAR cells (Figure 1b and Supplemental Table 3). The expression of NR1I3 (CAR), HNF4A, and RXRα was significantly decreased, but the other five TFs tested were unchanged (Figure 1c and Supplemental Table 4).

KD of ADARB1 significantly decreased the expression of most CYPs, except for CYP3A4, CYP2C9, CYP2B6, CYP1A2, and CYP1B1 (Figure 1a and Supplemental Table 2). CES2 mRNA expression was increased. Only UGT2B4 and UGT2B10
decreased (Figure 1b and Supplemental Table 3), and except for ARNT, the expression of all TFs changed (FOXA2 and RXRA increased while others decreased) in siADARB1 cells (Figure 1c and Supplemental Table 4). We validated the effects on CYP3A4 expression at the protein level, showing significantly increased CYP3A4 protein levels after KD of ADAR but not ADARB1 (Supplemental Figure 3). These results indicate that ADAR and ADARB1 have gene-specific effects, some occurring in opposing directions.

KD of ADAR or ADARB1 impacts the induction of CYPs and UGTs

We then tested the induction of CYP enzymes after inducer treatment in the ADAR and ADARB1 KDs. Three inducers were used: rifampicin (functions through PXR/NR1I2), 3-Methylcholanthrene (3MC) (via AHR), and CITCO (via CAR/NR1I3). Unexpectedly, 3MC treatment decreased the expression of ADARB1 in the control and siADARB1 cells. Other agonist treatments did not affect ADAR or ADARB1 expression (Supplemental Figure 1b). As shown in Supplemental Table 2, rifampicin induced the expression of the CYP3As, CYP2Cs, CYP2A6, and CYP2B6, as expected (Chen and Raymond, 2006). After 3MC treatment, CYP1A2 and CYP1B1 markedly increased, as previously reported (Pansoy et al., 2010), but expression of all other CYPs decreased. As expected, CITCO increased the expression of CYP2B6 by ~10-fold and all other CYPs by ~2-fold (excluding CYP2E1).

We used two-way ANOVA to analyze the interactions between KD of the ADARs and agonist treatment on CYP expression. Significant interactions indicate altered induction fold-change in the KDs compared to the control cells. We observed numerous statistically significant interactions between KD of the ADARs and inducer treatment (Figure 2 and Supplemental Table 2). CYP induction was either significantly reduced or
unaffected but not enhanced in the ADAR KD, while this occurred in both directions in the ADARB1 KD (Supplemental Table 2). The effects differ for each CYP-agonist pair and vary between the siADAR and siADARB1 cells. For example, induction of CYP3A4 (by rifampicin) and CYP2B6 (by CITCO) was reduced in siADAR but not in siADARB1 (Figure 2). ADAR KD reduced the induction of CYP2C9 and CYPC19, but not CYP2C8, while ADARB1 KD increased CYP2C8 induction but did not affect CYP2C9 or CYP2C19. Similarly, induction of CYP2A6 by rifampicin was slightly increased in siADARB1 cells, while induction of CYP2B6 was reduced in the ADAR KD (Figure 2 and Supplemental Table 2). In general, KD of ADAR more broadly impacted induction of the CYPs (9/12, five of which were affected in ≥ 2 treatments) than ADARB1 (6/12, four of which in ≥ 2 treatments) (Supplemental Table 2). These results further emphasize that ADAR and ADARB1 differentially regulate the expression of CYP enzymes.

We also tested the induction of UGTs. All three inducers induced the UGT1As, while rifampicin and CITCO induced UGT2B4. Overall, even when significant, the magnitude of induction of the UGTs (<3-fold) was less than the CYPs (Supplemental Table 3). While KD of either ADAR or ADARB1 generally did not affect the basal expression of the UGT1As, there were several cases where induction was slightly reduced after KD ADAR or ADARB1: UGT1A1 (ADAR and ADARB1), UGT1A4 and UGT1A6 (ADAR), and UGT1A3 and UGT1A9 (ADARB1) (Supplemental Table 3). siADARB1 cells had slightly increased induction of the UGT2Bs, while KD of ADAR did not alter UGT2B induction. Considering its effects on both basal and induced expression of the UGTs, ADARB1 appears to play a more prominent role in UGT expression than ADAR.
Investigating the associations between the expression of the CYPs, ADAR, ADARB1, and TFs in human liver samples

We measured the expression of ADAR-201 (encodes ADARp110), ADAR-202 (encodes ADARp150), and ADARB1 in a liver sample cohort (n=246). There was considerable variability in the expression levels of ADAR and ADARB1 between individuals, consistent with a previous report (Nakano et al., 2016) and gene expression data from GTEx (Carithers et al., 2015). The difference between the highest and lowest expressing samples for each isoform was 88-fold for ADAR-201, 98-fold for ADAR-202, and 191-fold for ADARB1. The expression level of ADAR-201 was the highest, followed by ADARB1 and ADAR-202 (Figure 3). There was a moderate correlation between levels of the two ADAR isoforms (r=0.485, P=6.8x10^{-16}) and ADAR-201 and ADARB1 (r=0.580, P=2.2x10^{-16}), but a low correlation between ADAR-202 and ADARB1 (r=0.232, P=2.4x10^{-4}).

We then tested for an association between the expression of the CYPs, critical TFs, and ADARs in the liver (adjusted for reported sex, race, and age). For this, we combined the expression of the two ADAR isoforms to account for the total pool of ADAR in the liver samples and obtained expression data for CYPs and TFs from our previous study (Collins and Wang, 2021). Except for CYP3A7 and CYP2A6, all CYPs and TFs tested were significantly associated with ADAR in a positive direction (Table 1). Similarly, except for CYP3A4, all CYPs and TFs were significantly associated with ADARB1, but the association directions were either positive (CYP3A5, CYP2C8, CYP2C9, CYP2C19, CYP2B6, CYP2E1, CYP1A2, all tested TFs) or negative (CYP3A7,
CYP2A6). The associations between the TFs and ADARs were generally stronger than the CYPs and ADARs.

Using a multivariate regression model that included the TFs as covariates, all associations between the CYPs and ADAR became insignificant, except for CYP2C8 and CYP3A5 in CYP3A5*3 homozygotes (further discussed below) (Table 2). Similarly, most associations between the CYPs and ADARB1 became insignificant, except for negative associations with CYP3A4, CYP3A7, CYP2A6, and CYP2E1. These results support that the effects of the ADARs on CYP expression may primarily be indirect through TFs, but direct and independent effects of the TFs cannot be excluded.

KD of ADARs changed the aberrant CYP3A5 splicing associated with CYP3A5*3

CYP3A5*3 creates a cryptic splicing site and produces an aberrant transcript that undergoes nonsense-mediated decay (Busi and Cresteil, 2005), resulting in decreasing the expression of normally-spliced CYP3A5 (CYP3A5-NOR) to ~1/8 of *1 in liver samples (Collins and Wang, 2021). Interestingly, and differently than the other CYP3As, CYP3A5-NOR was significantly and negatively associated with levels of ADAR after adjusting for TFs in CYP3A5*3 homozygotes but not in CYP3A5*1 carriers (Table 2). This prompted us to consider that ADAR may promote the aberrant splicing of CYP3A5*3, causing decreased expression of the normal CYP3A5 transcript in CYP3A5*3 carriers. To test this, we measured the ratios of normal CYP3A5 and aberrant CYP3A5*3 transcripts (CYP3A5_AB) (CYP3A5-NOR/CYP3A5-AB) in control and both ADAR and ADARB1 KDs. HepaRG cells are homozygous for CYP3A5*3 (Jackson et al., 2016). Thus, all detectable CYP3A5-NOR transcripts “escaped” the effects of CYP3A5*3. The ratio of CYP3A5-NOR/CYP3A5-AB significantly increased in
siADAR (1.4-fold) and siADARB1 (1.6-fold) compared to the control (Figure 4). These results indicate that the ADARs promote using the cryptic splice site generated by CYP3A5*3.

Discussion

In this study, using siRNAs targeting either ADAR or ADARB1 in HepaRG cells, we found that KD of ADAR or ADARB1 broadly changed the basal and induced expression of CYPs and UGTs in HepaRG cells. The effects were in both directions but mainly caused repression and differed between ADAR and ADARB1 (Figure 1 and Supplemental Table 2). Consistent with the KD experiments, the expression of most CYPs tested was significantly associated with ADAR and ADARB1 in the expected direction in liver samples (Table 1). Overall, our data support both broad and differential roles for ADAR and ADARB1 in maintaining the expression of the CYPs and UGTs in the liver and that they may broadly influence drug metabolism and pharmacokinetics for many drugs, as previously suggested (Nakano and Nakajima, 2018).

Numerous TFs are known to coregulate the expression of the CYPs (Collins and Wang, 2021). Previous studies indicate that the effects of ADAR and ADARB1 may be primarily mediated through HNF4A, PXR, CAR, and AHR (Nakano et al., 2016; Nakano et al., 2019; Nozaki et al., 2019; Takemoto et al., 2021). We found that the expression of CAR, RXRA, and HNF4A was reduced by KD of the ADARs (Figure 1c), and the associations between TFs and ADARs were stronger than CYPs and ADARs in the liver samples (Table 1). Including the TFs as covariates in the regression models abolished most associations between the CYPs and ADARs (Table 2), supporting that the effect of the ADARs on CYP expression is primarily indirect.
However, TF-independent effects also exist; for example, KD of ADAR increased the expression of the CYP3As and CYP2C9, which is inconsistent with decreased expression of CAR, RXRA, and HNF4A in siADAR cells. Our results suggest that this may be a general phenomenon for the CYP3As, as expression of both CYP3A7 and CYP3A43 were also elevated.

ADAR and ADARB1 can regulate gene expression through different pathways in editing-dependent or editing-independent manners, affecting splicing, mRNA stability, miRNAs, etc. (Wang et al., 2013; Solomon et al., 2017). Taking aberrant splicing of CYP3A5*3 as an example, we detected a potential role for the ADARs in promoting the usage of the CYP3A5*3 cryptic splice site (Figure 4), thereby reducing normal CYP3A5 transcripts in CYP3A5*3 carriers (Table 2). Numerous A-to-I editing sites nearby the CYP3A5*3 cryptic splicing site are reported in the REDIportal database (Picardi et al., 2017), supporting the direct interaction of these proteins with the CYP3A5 transcript. The CYP3A genomic sequences are highly homologous with each other, and all CYP3As contain A-to-I editing sites (Picardi et al., 2017). Therefore, the effects of the ADARs on CYP3A5 may extend to all of the CYP3As. Since CYP3A5*3 is a common allele and expression of the ADARs is highly variable between individuals and disease states, further experiments to elucidate the potential ADAR regulation of CYP3A5*3 splicing and normal transcript expression are warranted.

We also identified CES1 and CES2 as additional drug-metabolizing enzymes altered by KD of ADAR or ADARB1, extending ADARs regulation to the carboxylesterases. KD of ADAR did not primarily affect the UGTs, but ADARB1 significantly reduced the expression of both UGT2B4 and UGT2B7, indicating that the
ADARs may regulate the expression of additional UGT family members, as well (Figure 1b & Supplemental Table 2).

In general, our experiments illustrated that KD of the ADARs altered the induction of the CYPs, although the effects were not uniform across all CYPs. This is not entirely unexpected, however, as the agonists are known to bind and coordinate the activity of multiple TFs that ultimately mediate their effects (Abdelrahim et al., 2006; Chen and Raymond, 2006; Rasmussen et al., 2017; Lin et al., 2020). Also, agonist-mediated induction was mostly weak or moderate (2-3 fold) for those CYPs that were not the primary targets (e.g., rifampicin’s impact on CYP3A4 versus CYP2C9). Therefore, the role of the ADARs in regulating these other TF targets may have been harder to detect. Since our experiments measured the changes in the mRNA expression of the TFs, we cannot exclude that additional ADAR regulation at the protein level may be influencing CYP induction. Indeed, there was a trend for reduced induction of all genes after ADAR KD. Conversely, KD of ADARB1 both increased and reduced induction, indicating it has a more complex role in CYP regulation. In support of this, the 3’-UTR of CYP2B6 is a direct target of ADARB1, which stabilizes the CYP2B6 transcript, promoting its expression (Nozaki, 2019). Due to this, it may be difficult to fully disentangle the role of ADARB1 in controlling the expression of the CYPs, and more focused efforts to detect direct ADARB1 impacts are warranted.

Our siRNA experiments largely agreed with previous experiments in different hepatic cell lines, namely that most CYPs have reduced expression after ADAR KD, except for CYP3A4 (Nakano et al., 2016; Nakano et al., 2019; Nozaki et al., 2019; Takemoto et al., 2021). However, discrepancies do exist. In HepG2 cells, PXR
increases after KD of ADAR, and it was suggested that this led to higher CYP3A4 expression (Takemoto et al., 2021). However, we did not observe a change in PXR expression after ADAR KD in HepaRG cells. Instead, siADARB1 cells had decreased expression of both PXR and the CYP3As (excluding CYP3A5) (Figure 1a). Paradoxically, rifampicin-mediated induction was slightly enhanced in siADARB1 cells and suppressed in siADAR cells (Figure 2 and Supplemental Tables 2 and 3). Therefore, our results do not support PXR as the primary driver of increased CYP3A4 expression after ADAR KD.

Unlike the results in Huh-7 cells, where AHR expression increases after ADAR KD and enhances the induction of CYP1A1 by AHR agonists (Nakano et al., 2016), we did not observe a change in the induction of CYP1A2 or CYP1B1 in response to 3MC (Supplemental Table 2). Instead, we observed repressive effects of 3MC on all CYPs tested, except for CYP1A2, CYP1B1, and the UGTs (Supplemental Tables 2 and 3), possibly caused by competition between AHR and PXR (Rasmussen et al., 2017), and in HepaRG cells, AHR is expressed much higher than PXR (>50 fold), which may explain the strong suppression of CYP expression by 3MC. Similarly, we observed lower CAR expression and reduced CITCO-mediated induction after KD ADAR (Figure 1c) in HepaRG cells, which differs from the results in HepG2 cells showing increased CAR expression and CYP2B6 induction (Nakano et al., 2019).

These apparent discrepancies may be due to the following reasons. Firstly, there are known differences in gene expression between hepatic cancer lines (Jennen et al., 2010) and specifically in the expression of the drug-metabolizing enzymes (Guo et al., 2011) and their TFs (Kanebratt and Andersson, 2008). Secondly, TFs have numerous
splice isoforms with different activity and expression (see review by Joung et al., 2023), and primers used in qPCR may capture and detect diverse TF splice isoform populations. Finally, the previous experiments noted that KD of ADAR also reduced ADARB1 (Nozaki et al., 2019; Takemoto et al., 2021), and their non-overlapping effects may have influenced the previous results. In contrast, we did not observe cross-ADAR effects upon KD of either ADAR isoform (Supplemental Figure 1).

A limitation of our study is that the results are primarily based on mRNA data and do not capture ADAR impacts at the protein level. As the ADARs are known to influence nearly all stages of gene expression, additional ADAR effects on drug metabolism likely occur post-transcriptionally that are not identified here. For example, while our protein measurements of CYP3A4 support its increased expression after ADAR KD, we did not observe reduced CYP3A4 in siADARB1 cells (Supplemental Figure 3). This conflicting result may be caused by additional post-transcriptional effects on CYP3A4 expression that occur after KD of ADARB1. Alternatively, it may be due to the relatively minor change in CYP3A4 mRNA expression after ADARB1 KD (<1.5-fold) that is undetectable at the protein level. Also, while our study does not provide mechanistic insight into how the ADARs regulate the CYPs, we believe that they contribute to the growing body of literature supporting the wide-spread regulatory roles of the ADARs, and, more specifically, their involvement in the regulation of the drug-metabolizing enzymes in the liver.

In summary, our data support the widespread role of ADAR and ADARB1 in regulating the expression of the drug-metabolizing enzymes in the liver. ADARs are upregulated in neurological diseases and many cancers (Qi et al., 2014; Gallo et al., 2017;
Li et al., 2021; Baker and Slack, 2022; Song et al., 2022), and ADAR inhibitors have been suggested as a targeted therapy for some cancers (Bhate et al., 2019). Our results indicate that factors that alter the expression or activity of the ADARs (e.g., genetic variation, disease states, inhibitors) likely will also change the basal and induced expression of the drug-metabolizing enzymes and broadly affect drug metabolism and drug-drug interactions. Furthermore, given their overall effects on gene expression, ADAR and ADARB1 may also regulate the expression of other pharmacogenes. Unraveling how the ADARs regulate pharmacogene expression will uncover factors controlling inter-person variability in drug metabolism and disposition.

Data Availability Statement
The authors declare that all the data supporting the findings of this study are contained within the paper.

Authorship Contributions
Participated in research design, experimentation, data analysis, and manuscript writing: Collins, J and Wang, D.

References


Visualization—EBI GBDI, and Genome Browser Data Integration


Footnotes

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Conflict of interest statement

No author has an actual or perceived conflict of interest with the contents of this article.
Figure legends

Figure 1. Impact of ADAR and ADARB1 KD in HepaRG cells on the expression of CYPs and CESs (a), UGTs (b), and TFs (c). Plots show the average ddCT value of each gene in each ADAR KD (n=3), calculated as ddCT(KD – Control). Compared to the controls, ADAR KD values are square and colored blue if significant (P<0.05), while ADARB1 KD values are triangular and colored red if significant (P<0.05). The dashed line centered on zero indicates the expression of the control. The dotted lines indicate ± 1.5 fold-difference between the KD and control. Detailed gene expression data are shown in Supplemental Tables 2-4.

Figure 2. Induction of select CYP enzymes in siADAR (blue, upper panels) and siADARB1 (red, lower panels) KDs. Estimated marginal means and 95% confidence intervals are displayed for each KD/treatment combination (n=3). Solid lines indicate significant interactions between KD and treatment, as determined by two-way ANOVA (P<0.05). Gene expression is shown as dCT values (β-actin – gene). All tested genes' detailed induced gene expression data are shown in Supplemental Tables 2 and 3.

Figure 3. Expression of the two ADAR transcripts (ADAR-201 and ADAR-202) and ADARB1 in human liver samples (n=246). Violin plots show the distribution of gene expression, with internal boxplots showing median and IQR and whiskers showing 1.5*IQR. ADAR mRNA expression was normalized to β-actin and is shown as dCT^2*100000.

Figure 4. Changes in the ratios of CYP3A5 normal (CYP3A5-NOR) to aberrant (CYP3A-AB) transcripts in the ADAR KD cells. Mean and SEM are superimposed on individual expression values (n=3). *P<0.05, **P<0.01.
Table 1. Association between CYPs, TFs, and ADAR or ADARB1 expression levels in liver samples.

<table>
<thead>
<tr>
<th>Gene</th>
<th>ADAR1 coefficient</th>
<th>ADAR1 P value</th>
<th>ADARB1 coefficient</th>
<th>ADARB1 P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP3A4</td>
<td>0.631</td>
<td>0.001</td>
<td>0.071</td>
<td>0.585</td>
</tr>
<tr>
<td>CYP3A5</td>
<td>0.676</td>
<td>1.35x10^-5</td>
<td>0.323</td>
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</tr>
<tr>
<td>3A5*3 GG</td>
<td>0.524</td>
<td>0.01906</td>
<td>0.359</td>
<td>0.016</td>
</tr>
<tr>
<td>3A5*3 GA/AA</td>
<td>0.566</td>
<td>0.001</td>
<td>0.167</td>
<td>0.152</td>
</tr>
<tr>
<td>CYP3A7</td>
<td>0.107</td>
<td>0.672</td>
<td>-0.382</td>
<td>0.024</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>0.682</td>
<td>1.73x10^-5</td>
<td>0.365</td>
<td>7.56x10^-4</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>0.896</td>
<td>9.66x10^-9</td>
<td>0.493</td>
<td>2.87x10^-6</td>
</tr>
<tr>
<td>CYP2C8</td>
<td>1.124</td>
<td>1.97x10^-11</td>
<td>0.564</td>
<td>7.59x10^-7</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>0.785</td>
<td>4.82x10^-5</td>
<td>0.505</td>
<td>1.00x10^-4</td>
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<tr>
<td>CYP2A6</td>
<td>0.105</td>
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<td>-0.525</td>
<td>6.17x10^-4</td>
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<tr>
<td>CYP2E1</td>
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<td>3.73x10^-10</td>
<td>0.261</td>
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<tr>
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<td>2.31x10^-6</td>
<td>0.366</td>
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<tr>
<td>ESR1</td>
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<td>2.40x10^-7</td>
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<td>2.75x10^-4</td>
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<tr>
<td>RXRA</td>
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<tr>
<td>PXR</td>
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<td>8.60x10^-10</td>
<td>0.5652</td>
<td>4.91x10^-11</td>
</tr>
<tr>
<td>CAR</td>
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<td>2.12x10^-8</td>
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<td>1.93x10^-6</td>
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<tr>
<td>HNF4A</td>
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<tr>
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<tr>
<td>FOXA2</td>
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<tr>
<td>AHR</td>
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<td>ARNT</td>
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<td>9.70x10^-8</td>
<td>0.4633</td>
<td>1.30x10^-11</td>
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</tbody>
</table>

Adjusted by sex, age, and race
Table 2. Association between the expression levels of CYPs and ADAR or ADARB1 after adjusting for key TFs.

<table>
<thead>
<tr>
<th>Genes</th>
<th>ADAR1 coefficient</th>
<th>ADAR1 P value</th>
<th>ADAR1 coefficient</th>
<th>ADAR1 P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP3A4</td>
<td>-0.058</td>
<td>0.69</td>
<td>-0.3882</td>
<td>1.02x10^-04</td>
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<tr>
<td>CYP3A5</td>
<td>-0.165</td>
<td>0.102</td>
<td>-0.0994</td>
<td>0.131</td>
</tr>
<tr>
<td>3A5*3 GG</td>
<td>-0.251</td>
<td>0.036</td>
<td>-0.1557</td>
<td>0.057</td>
</tr>
<tr>
<td>3A5*3 GA/AA</td>
<td>-0.041</td>
<td>0.729</td>
<td>-0.0497</td>
<td>0.502</td>
</tr>
<tr>
<td>CYP3A7</td>
<td>0.007</td>
<td>0.98</td>
<td>-0.403</td>
<td>0.0377</td>
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<tr>
<td>CYP2C9</td>
<td>0.064</td>
<td>0.619</td>
<td>0.0293</td>
<td>0.75</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>0.279</td>
<td>0.058</td>
<td>0.06</td>
<td>0.559</td>
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<tr>
<td>CYP2C8</td>
<td>0.476</td>
<td>2.10x10^-05</td>
<td>0.1554</td>
<td>0.054</td>
</tr>
<tr>
<td>CYP2B6</td>
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<td>0.100</td>
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<tr>
<td>CYP2A6</td>
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<tr>
<td>CYP2E1</td>
<td>0.185</td>
<td>0.147</td>
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<td>3.30x10^-06</td>
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<tr>
<td>CYP1A2</td>
<td>0.293</td>
<td>0.094</td>
<td>-0.166</td>
<td>0.176</td>
</tr>
</tbody>
</table>

Adjusted by sex, age, race, and TFs
A comprehensive evaluation of the effects of RNA-editing protein ADAR and ADARB1 on the expression of the drug-metabolizing enzymes in HepaRG cells. Joseph M. Collins and Danxin Wang. Drug Metabolism and Disposition. DMD-AR-2023-001396D

Supplemental Figure 1

Supplemental Figure 1. ADAR expression after siRNA and inducer treatment. a) Expression of ADAR and ADARB1 mRNA after KD of either ADAR (blue) or ADARB1 (red). ***P<0.001. b) Expression of the ADAR and ADARB1 after KD and agonist treatment. Estimated marginal means and 95% confidence intervals are displayed for each KD/agonist combination (n=3). All expression data are shown after normalization to B-actin mRNA expression. **P<0.01, compared to DMSO treatment.
Supplemental Figure 2. ADAR and ADARB1 protein expression after siRNA treatment. Protein levels in the control and both KDs were detected with a) anti-ADAR, b) anti-ADARB1, and c) anti-B-actin antibodies. Arrows indicate the expected bands for each protein. The asterisk indicates a known smaller isoform of ADARB1 detected by the anti-ADARB1 antibody, as reported by Proteintech®. ADAR and ADARB1 were detected using anti-mouse-HRP, and B-actin was detected using anti-mouse-NIR.
Supplemental Figure 3. CYP3A4 protein expression in the control, siADAR and siADARB1 HepaRG cells. A) Example western blot image showing CYP3A4 and B-actin expression in the HepaRG cells. Arrows indicate expected protein band sizes. CYP3A4 and B-actin were detected using anti-mouse-HRP. B) Quantified protein levels in the samples. CYP3A4 protein levels were normalized by measured B-actin. Individual replicates are shown (n=3), and mean and SEM are superimposed on individual expression values. Student’s t-test **P<0.01, ns non-significant.