**ABSTRACT**

This is a report of a symposium on the potential role of epigenetic mechanisms in the control of drug disposition sponsored by the American Society for Pharmacology and Experimental Therapeutics and held at the Experimental Biology 2013 meeting in Boston, MA, April 21, 2013. Epigenetics is a rapidly evolving area, and recent studies have revealed that expression of drug-metabolizing enzymes and transporters is regulated by epigenetic factors, including histone modification, DNA methylation, and noncoding RNAs. The symposium speakers provided an overview of genetic and epigenetic mechanisms underlying variable drug metabolism and drug response, as well as the implications for personalized medicine. Considerable insight into the epigenetic mechanisms in differential regulation of the dioxin-inducible drug and carcinogen-metabolizing enzymes CYP1A1 and 1B1 was provided. The role of noncoding microRNAs in the control of drug metabolism and disposition through targeting of cytochrome P450 (P450) enzymes and ATP-binding cassette membrane transporters was discussed. In addition, potential effects of xenobiotics on chromatin interactions and epigenomics, as well as the possible role of long noncoding RNAs in regulation of P450s during liver maturation were presented.

**Introduction**

Drug absorption, distribution, metabolism, and excretion (ADME) are critical processes that must be understood for the development of safe drugs. These processes are mediated by drug-metabolizing enzymes and transporters that are expressed in various tissues, including the small intestine, liver, and kidney. These processes can limit or enhance the systemic and target organ exposure to xenobiotics. In particular, drug-metabolizing enzymes such as cytochrome P450 (P450) isoforms govern the metabolic elimination of drugs, and membrane transporters such as ATP-binding cassette (ABC) transporters can affect drug absorption, distribution, and excretion processes. Thus the interplay of drug-metabolizing enzymes and transporters may determine the pharmacokinetic properties of a drug such as bioavailability, volume of distribution, and half-life, and understanding the regulation of drug-metabolizing enzymes and transporters is necessary for the prediction of consequent pharmacological and toxicological effects.

There are considerable variations in drug metabolism and transport in humans (Ingelman-Sundberg et al., 2007; Yu, 2009; Giacomini et al., 2010; Yokoi and Nakajima, 2013) that can alter drug efficacy or cause adverse drug reactions. The latter is a leading cause of morbidity and mortality during pharmacotherapy. Indeed, the expression of ADME genes is tightly controlled by a variety of molecular regulatory mechanisms, among them transcription factors and cellular processes such as membrane trafficking and subcellular organization (Correia and Liao, 2007; Klaassen and Aleksunes, 2010). Genetic polymorphisms of ADME genes are also recognized for their clinical significance. Furthermore, concurrent use of drugs may lead to drug–drug interactions (DDIs) through the inhibition of enzyme or transport functions, or the alteration of ADME gene expression. Nevertheless, the interindividual differences in expression of the ADME genes are...
tremendously large and this variation cannot be solely explained by
these factors. Additional mechanisms such as the more recently identified
epigenetic factors warrant investigation in relation to ADME processes.
The symposium was organized to highlight and stimulate studies on such
epigenetic factors, including DNA methylation, histone modification, and
noncoding RNA (ncRNA) –mediated post-transcriptional regulation
(Gomez and Ingelman-Sundberg, 2009b; Nakajima and Yokoi, 2011;
Yu and Pan, 2012; Yokoi and Nakajima, 2013), studies which are expected
to enhance mechanistic understanding of variable pharmacokinetics and multidiug resistance, and provide novel insights into
individualized medication.

A symposium sponsored by the American Society for Pharmacology and Experimental Therapeutics was held at the Experimental Biology Annual Meeting in Boston, MA, April 21, 2013. Presentations were given by M.I-S., X.-B.Z., O.H., and A.-M. Y. to overview and exemplify the potential role of epigenetic regulatory mechanisms in cellular drug metabolism and transport, consequent effects on pharmaco-
kinetics and pharmacodynamics, and implications for improved pharmacotherapy. In addition, one abstract relating to long noncoding RNAs (lncRNAs) was presented by L.P. at the symposium. This article summarizes the presentations.

Overview of Genetic and Epigenetic Mechanisms Underlying Variable Drug Metabolism and Drug Response (M.I-S.)

Interindividual differences in expression of genes responsible for drug metabolism, transport, and response are caused by genetic, epigenetic, physiologic, and environmental factors. Much information has evolved regarding the role of genetic polymorphism in such variability. The number of genetic biomarkers for prediction of drug dosage and choice are increasing. Recent genome-wide association studies reveal that polymorphisms among the drug-metabolizing enzymes affect endogenous functions such as blood pressure, suicide risk, and bilirubin levels, as well as exogenous factors like coffee intake, cigarette consumption, and drug efficacy (Sim et al., 2013). Such polymorphisms and others, including variations in the human leukocyte antigen (HLA) system, as well as the levels of expression of different tyrosine kinases and other signal transduction polymorphisms, provide together an arsenal of pharmacogenomic biomarkers that can help optimize drug treatment, particularly in the areas of oncology, cardiovascular disease, infection, and psychiatry.

The epigenetic causes for variation in drug metabolism and response have been much less studied. A novel method for target enrichment–based analyses of 174 ADME genes in fetal and adult livers was presented (Ivanov et al., 2013). Genomic DNA from fetal and adult livers were sheared and subjected to Agilent Sure select target enrichment for the ADME genes, followed by next generation sequencing using protocols with or without bisulfite treatment (Kacevska et al., 2012). In total 0.5 million cytosine-phosphate (CpG) islands were analyzed. In general the variability in DNA methylation of the ADME genes between liver samples was conserved, but at some DNA regions variable methylation was seen. The extent of total methylation variation between different ADME genes was very different and occurred primarily in the open reading frames. An in depth analysis of the CYP3A4 gene revealed that specific elements in the 5’ upstream region were highly methylated in fetal livers, where no expression of the gene occurs, but severely decreased in adult livers, which suggested the importance of the higher CYP3A4 gene expression in this organ. Some DNA elements in the CYP3A4 gene were much conserved with respect to methylation in the adult livers, whereas others were very variable. The methylation of specific CpG sites correlated with the expression of the CYP3A4 gene as analyzed at the transcriptomic level (Kacevska et al., 2012a).

We found that besides methylation of cytosine, 5-hydroxymethylation is indeed an important modification of cytosine in liver (Ivanov et al., submitted manuscript). Thus, we found using mass spectrometry that up to 1% 5-hydroxymethylcytosine (5hmC) of the total cytosine content in adult liver consists of 5hmC, whereas in fetal livers it is below 0.125%. Genome-wide mapping of the distribution of 5hmC in human adult liver samples showed that 5hmC occupancy was over-represented in genes involved in active catabolic and metabolic processes, whereas 5hmC elements that were found in genes from fetal livers were not present in the adult state. Overall we found a localization of 5hmC in enhancers and in CpG islands and in the active genes, and that the amount of 5hmC was indeed high. The data emphasize the importance of using methods that permit the discrimination between 5mC and 5hmC when investigating the liver epigenome, as methods based on bisulphite sequencing will cause erroneous conclusions.

It was emphasized that altered gene methylation is important for creating drug resistance in tumors during anticancer treatment and that the progress of tumor removal and treatment can be monitored by quantification of tumor-specific methylation of circulating DNA. This indicates the possibility of following tissue-specific epigenetic alterations in circulating blood. It was concluded that much more research is needed before the mechanisms behind and the role of gene methylation for drug metabolism (Fig. 1), transport, and action can be understood.

Role of Epigenetic Mechanisms in Differential Regulation of the Dioxin-Inducible Human CYP1A1 and CYP1B1 Genes (O.H. and S.B.)

The human CYP1A1 and CYP1B1 genes are highly inducible by agonists of the aryl hydrocarbon receptor (AHR), including 2,3,7,8-tetrachlorodibenzo-p-dioxin (dioxin) and certain polycyclic aromatic hydrocarbons (PAHs), such as benzo(a)pyrene. Induction of these genes involves the following steps: activation of the AHR by agonists such as dioxin, subsequent translocation of AHR to the nucleus, its dimerization with the aryl hydrocarbon receptor nuclear translocator, the binding of the AHR/aryl hydrocarbon receptor nuclear translocator dimer to xenobiotic response elements (XREs) located in the upstream enhancer regions of the above genes, the recruitment of coactivator proteins to the regulatory regions of the genes, the binding of general transcription factors to the promoter, followed by recruitment of the TATA-binding protein (TBP) and RNA polymerase II to the promoter, and then activation of gene transcription. CYP1A1 and CYP1B1 are particularly effective at metabolically activating polycyclic aromatic hydrocarbons, carcinogenic components prevalent in cigarette smoke, smog, and cooked foods. It is well established that methylation of cytosine residues of genomic DNA (DNA methylation) of the regulative region(s) of a gene can lead to silencing or diminished expression of the gene. Of particular interest, the promoter of the CYP1B1 gene exhibits DNA methylation in a portion of colorectal (Habano et al., 2009) and gastric (Kang et al., 2008) cancers. Studies conducted in our laboratory demonstrated the mechanisms through which DNA methylation causes CYP1B1 silencing and also shed light on the mechanisms of gene regulation in mammalian cells in general (Beedanagari et al., 2010a).

Our studies emanated from our initial observations that CYP1A1 mRNA is highly induced by dioxin in both the human breast cancer cell line MCF-7 and the human hepatoma cell line HepG2, but whereas CYP1B1 mRNA is induced in MCF-7, it is not induced in
HepG2 cells. The *CYP1B1* promoter abutting the transcriptional start site, and the gene’s upstream enhancer region (which contains XREs to which the liganded AHR can bind) have a high density of CpG dinucleotides, representing CpG “islands.” Bisulfite sequencing revealed that 22 of the 24 CpG sites within 280 bp encompassing the promoter region of *CYP1B1* (–260 to +20) are fully methylated in HepG2 cells, whereas only one of the 24 CpG sites was methylated in MCF-7 cells. The CpG sites in a 360-bp sequence (–560 to –920 bp) encompassing the enhancer of *CYP1B1* were either only partially methylated (0–60%) or not methylated in HepG2 cells, while these CpG sites in MCF-7 cells were not methylated. There are two XREs in the *CYP1B1* enhancer region (Fig. 2). The XRE sequence contains a CpG site, methylation of which is known to interfere with AHR binding. DNA methylation analysis of these XREs located in the *CYP1B1* enhancer region revealed that they are only partially methylated (30%) in HepG2 cells, but not methylated in MCF-7 cells. Treatment of HepG2 cells with the DNA methyltransferase inhibitor 5-azadC reactivated dioxin-induced *CYP1B1* mRNA expression to a level comparable to that in MCF-7 cells. Furthermore, 5-AzadC treatment led to significant demethylation of the CpG sites in the *CYP1B1* promoter and enhancer regions in HepG2 cells. Collectively, these data indicate that DNA methylation directly inhibits dioxin inducibility of the *CYP1B1* gene in HepG2 cells.

We investigated the consequences of DNA methylation of the *CYP1B1* gene. Using the chromatin immunoprecipitation assay, we demonstrated that dioxin treatment fails to lead to the recruitment of AHR to the enhancer region of the *CYP1B1* gene in HepG2 cells. However, these recruitments were restored after 5-AzadC treatment. These observations are consistent with the observation that CYP1B1 mRNA inducibility is reactivated in this cell line by 5-AzadC. Importantly, dioxin treatment led to recruitment of AHR to the enhancer region of the *CYP1B1* gene in HepG2 cells. It is known that the transcriptional coactivator p300 is required for maximal dioxin induction of *CYP1B1* (Taylor et al., 2009). We demonstrated that dioxin treatment led to recruitment of p300 and the coactivator p300/CBP-associated factor (PCAF) to the enhancer of *CYP1B1* in HepG2 cells. Thus loss of dioxin inducibility of CYP1B1 in HepG2 cells due to DNA methylation is ascribable to events in the induction pathway subsequent to AHR, p300, and PCAF recruitment to the enhancer.

Chromatin modifications play an important role in the epigenetic regulation of the transcription of genes. Acetylations of histone H3 at lysines 9 and 14, acetylation of histone H4, and trimethylation of histone H3 at lysine 4 are generally associated with actively transcribed genes. We found that the above histone modifications were markedly increased at the *CYP1B1* promoter of MCF-7 cells after dioxin treatment, and that these increases were dependent upon p300 (which is capable of directly catalyzing the above acetylations). However, in dioxin-treated HepG2 cells, Pol II recruitment did not increase despite enhanced coactivator recruitment, implying a dissociation between this event and chromatin modification under these conditions. This is most likely due to DNA methylation at the promoter.

These studies therefore provided a detailed description of the mechanism whereby DNA methylation inhibits dioxin induction of the *CYP1B1* gene, many aspects of which may be applicable to other genes whose expression is modified by DNA methylation. The following of our observations are of particular interest: 1) Recruitment of AHR to the enhancer of the *CYP1B1* gene does not necessarily equate with dioxin inducibility, consistent with the authors’ other studies (Beedanagari et al., 2010b) and others (Yang et al., 2008). 2)
The binding of TBP to gene promoters generally seeds the association of other general transcription factors. Our observation that DNA methylation at the CYP1B1 promoter inhibits dioxin-induced binding of TBP suggests that this may represent a critical step in the process whereby DNA methylation at the promoter inhibits gene expression; and this may be the case for other genes subject to silencing by methylation. 3) Our observations indicate that p300 recruitment to the enhancer is not sufficient for the generation of histone modifications at the promoter, but that communication with other protein(s) at the promoter is probably required. 4) Transcriptional coactivators are generally incorporated in large multiprotein complexes straddling both the enhancer and promoter of responsive genes. We observed much greater levels of p300 and PCAF (as well as AHR) at the CYP1B1 enhancer than at the CYP1B1 promoter in MCF-7 cells treated with dioxin. This observation is consistent with the notion that these proteins are in closer proximity to AHR at the CYP1B1 enhancer than to TBP and the general transcription factors located at the promoter. It is therefore of considerable interest that p300 and PCAF are recruited efficiently at the CYP1B1 enhancer after dioxin treatment in HepG2 cells, despite the fact that TBP and Pol II are not recruited to the corresponding promoter. These observations strongly imply that p300 and PCAF can be recruited to the enhancer even when they are not incorporated into a multiprotein complex spanning the enhancer and the promoter (Fig. 2). The lack of a requirement for coactivators to be tethered at both the promoter and enhancer can probably be generalized to other coactivators and other genes, and this represents an important area for future research.

**Chromatin Interactions, Epigenomics, and Transcriptional Outcomes in Response to Xenobiotics (X.-B.Z.)**

DDI is a significant clinical concern especially for people at ages above 50 because ~78% of people over 50 currently using drugs actually take more than one drug a day, and 4% of them are at risk for either a reduced therapeutic efficacy or adverse drug reactions (Qato et al., 2008). One major cause of DDI is that some drugs have the ability to alter the expression of drug-metabolizing enzymes or transporters responsible for the ADME of coadministered drugs. It has been known that at the molecular levels ADME genes are induced through the activation of transcriptional factors such as nuclear receptor pregnane X receptor (PXR) and constitutive androstane receptor by the drugs (Urquhart et al., 2007). Upon activation by the drugs, the nuclear receptor binds to its response elements at multiple genomic locations to regulate the transcription of ADME genes. However, many questions remain to be answered for a thorough understanding of the mechanisms underlying the induction of ADME genes by drugs. For example, many nuclear receptor binding sites are located far away from the promoters of their target genes. How are those binding sites involved in the transcription regulation of their target genes? Are long-range chromatin interactions involved in bringing these binding sites close to their target promoters for possible interactions, and thus facilitating the transcriptional regulation? Are the regulatory elements acting alone or being coordinated in clusters in response to xenobiotics? What are the epigenomic features associated with ADME genes and regulatory elements?

Dr. Zhong from the University of Connecticut and his collaborators, Drs. Yijun Ruan and Guoliang Li from The Jackson Laboratory (Farmington, CT), intend to improve the understanding of molecular mechanisms of drug-induced change in ADME gene expression. They have selected rifampicin as a model drug in a planned study. Based on the literature and their preliminary data, they formed a central hypothesis that rifampicin activates the transcription factor PXR, which binds to its DNA response elements at multiple genomic locations and participates in long-range chromatin interactions in a three-dimensional conformation, thus helping to bring relevant genomic elements such as the enhancers and promoters into close spatial proximity for an efficient and coordinated transcription regulation. The changes in ADME gene expression further alter therapeutic efficacy of drugs coadministered with rifampicin. They will use human primary hepatocytes and established human liver cell lines (e.g., HepaRG for loss-of-function and HepG2 for gain-of-function) as in vitro models, and PXR-humanized mice as an in vivo model to test their hypothesis. They plan to define PXR-mediated chromatin interaction architecture, epigenomic features, and transcriptional outcomes in response to rifampicin in human liver cells. They will use computational and network approaches. The data are expected to provide a comprehensive repertoire of regulatory elements such as promoters and enhancers, and specific interaction networks of the regulatory functions in responding to rifampicin. Through the integrated analyses of multiplex datasets generated, they hope to understand how PXR activated by rifampicin participates in maintaining higher-order spatial proximity for an efficient and coordinated transcription regulation.
Noncoding MicroRNAs in the Control of Drug Metabolism and Transport (A.-M.Y)

In recent years there has been increasing interest in identifying ncRNAs and understanding their functions in regulation of cellular processes. A major portion (>96%) of the human genome comprises noncoding DNAs (Matera et al., 2007; Kung et al., 2013), which include ribosomal RNAs, transfer RNAs, small nucleolar RNAs, microRNAs (miRNAs), small interfering RNAs, P-element–induced wimpy testis–interacting RNAs, and lncRNAs. Among them the miRNAs, a class of small (18–25 nt in length) ncRNAs in the control of post-transcriptional regulation of target genes, represent one of the groups of ncRNAs most intensively studied toward the elucidation of chromatin structure and transcriptional outcomes, which would be helpful for the prediction of related interactions and development of proper strategies to prevent unwanted drug effects.

There is also increasing evidence supporting miRNA-controlled post-transcriptional regulation of ADME genes (Gomez and Ingelmann-Sundberg, 2009a; Yu, 2009; Nakajima and Yokoi, 2011; Yu and Pan, 2012; Yokoi and Nakajima, 2013), which were highlighted at the symposium. More and more studies have demonstrated that miRNAs are able to target the 3′-untranslated regions (3′UTRs) of mRNA targets. Over 2000 miRNAs have been identified in humans, and they are predicted to govern post-transcriptional regulation of thousands of protein coding genes in control of essentially all life processes.

TABLE 1

Some P450 drug-metabolizing enzymes and ABC transporters shown to be targeted by noncoding miRNAs

<table>
<thead>
<tr>
<th>MicroRNA</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1B1</td>
<td>miR-27b</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>miR-378</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>miR-27b, mmu-miR-298</td>
</tr>
<tr>
<td>ABCB1</td>
<td>miR-451, miR-27a</td>
</tr>
<tr>
<td>ABCG2</td>
<td>miR-520h, miR-519c, miR-328</td>
</tr>
<tr>
<td>ABCC1</td>
<td>miR-134, miR-326, miR-199a, miR-199b, miR-296, miR-1291</td>
</tr>
<tr>
<td>ABCC2</td>
<td>miR-379</td>
</tr>
<tr>
<td>ABCC3</td>
<td>miR-9-3p</td>
</tr>
<tr>
<td>ABCC4</td>
<td>miR-125a, miR-125b</td>
</tr>
<tr>
<td>ABCC5</td>
<td>miR-101, miR-125a, Let-7a</td>
</tr>
<tr>
<td>ABCC6</td>
<td>miR-9-3p</td>
</tr>
</tbody>
</table>
to determine whether lncRNAs are involved in the regulation of P450
ontogeny.

Male C57BL/6 mouse livers were collected at 12 different ages from prenatal, neonatal, adolescent to adult. Poly-T selected RNAs were sequenced and the expression levels of both protein-coding and
non-coding genes were determined. Approximately 15,000 genes, including ~2000 lncRNAs, were expressed in liver during maturation. About 70% of these lncRNAs were significantly changed across age
groups. Three major ontogenic expression patterns were identified for lncRNAs, and they fell into neonatal-, adolescent-, and adult-enriched
groups. The same patterns were also found for P450 genes. LncRNAs with potential roles in regulation of P450s ontogeny were initially
screened. In addition, we recognized one lncRNA that displayed an
inverse expression pattern with Cyp4b1 during liver maturation. With a
gene symbol Gm12839, this lncRNA is located approximately 40 kb
downstream of Cyp1b1 gene, and it was annotated as a pseudogene of
Cyp4b1. This lncRNA exhibits more than 90% sequence identity as
the 3’ end of Cyp4b1 RNA. As the 3’UTR usually contains regulatory
elements for the ncRNAs, it would be interesting to determine whether
Gm12839 RNA downregulates the expression of Cyp4b1. This is
the first attempt to examine the correlation of lncRNAs and P450
ontogeny in liver and should facilitate future study on the possible
role of lncRNAs in the regulation of P450 expression during liver
maturation.

Summary
In this symposium, there has been a growing interest of some of the
ongoing studies defining the important role of epigenetics in regulation
of drug-metabolizing enzymes and drug transporters. It is clear from
the presentations and the questions raised during discussion that further
studies on the role of gene expression in drug metabolism and disposition
are forthcoming and highly warranted.

Authorship Contributions
Performed data analysis: Ingelman-Sundberg, Zhong, Hankinson, Beeda-
nagari, Yu, Peng, Osaka.

Wrote or contributed to the writing of the manuscript: Ingelman-Sundberg, Zhong, Hankinson, Beedanagari, Yu, Peng, Osaka.

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