

# Chronopharmacology: New Insights and Therapeutic Implications

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## Abstract

Most facets of mammalian physiology and behavior vary according to time of day, thanks to endogenous circadian clocks. Therefore, it is not surprising that many aspects of pharmacology and toxicology also oscillate according to the same 24-h clocks. Daily oscillations in abundance of proteins necessary for either drug absorption or metabolism result in circadian pharmacokinetics, and oscillations in the physiological systems targeted by these drugs result in circadian pharmacodynamics. These clocks are present in most cells of the body, organized in a hierarchical fashion. Interestingly, some aspects of physiology and behavior are controlled directly via a “master clock” in the suprachiasmatic nuclei of the hypothalamus, whereas others are controlled by “slave” oscillators in separate brain regions or body tissues. Recent research shows that these clocks can respond to different cues and thereby show different phase relationships. Therefore, full prediction of chronopharmacology in pathological contexts will likely require a systems biology approach that considers chronointeractions among different clock-regulated systems.

## INTRODUCTION

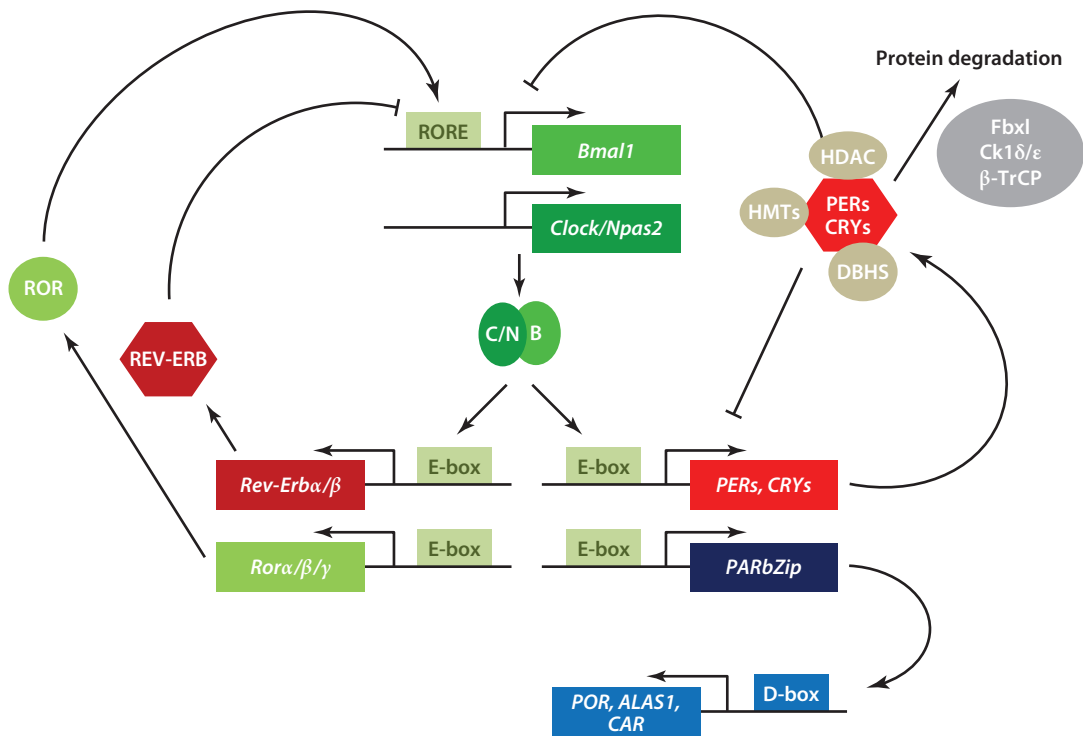
As a result of living on a planet whose principal source of light and heat is only periodically present, organisms on Earth rapidly adapted physiological systems to exploit these variations for maximum fitness. Collectively, these systems are named circadian clocks (from the Latin phrase *circa diem* meaning about a day). In mammals, circadian clocks influence all major organ systems, and this influence translates directly into disease pathology that also varies with time of day. Rhythmic physiology has long been recognized to result in rhythmic disease symptoms. Hippocrates noticed as early as 400 BC that daytime sleepiness is indicative of disease and nighttime sleeplessness can indicate pain and suffering (1). By medieval times, there were reports of daily variations in diseases such as bronchial asthma (2). For more than 30 years, it has been known that drug absorption and distribution are subjected to diurnal variation in rodents and humans. A 24-h change in drug bioavailability has therefore been established for hundreds of drugs. For example, acetaminophen (3) and theophylline (4) show different pharmacokinetics in the morning compared with the evening. These changes are the results of several time-dependent modifications of physiological and molecular processes that influence drug absorption and distribution.

Considering the wide scope of circadian (patho)physiology, it is logical that the pharmacokinetics and pharmacodynamics (PK/PD) of many drugs would be circadian and therefore that drug efficacy and safety profiles would also vary with time of day. Nevertheless, this variation is only seldom considered by clinicians, drug developers, and regulators. In part, this apathy may stem from a lack of insight into the molecular mechanisms governing this control. However, two decades of intensive research have uncovered a wealth of information not only about basic mechanisms of circadian clocks but also about how they interact with physiology and disease. Below, we review this knowledge on cellular and systems levels and then consider its implications for pharmaceutical intervention.

## MOLECULAR FUNDAMENTALS OF CIRCADIAN CLOCKS

The basic unit of circadian timekeeping is the cell: Even in complex organisms, most cells contain autonomous circuitry for circadian oscillations. Generally speaking, this mechanism is composed of negative feedback loops of transcription and translation: Activation of a repressor gene results in its later repression by its own protein product, and the instability of this repressor ensures that this repression is short-lived so a new cycle can begin. In mammals, the principal activators within this system are the CLOCK (circadian locomotor output cycles kaput) and BMAL1 (brain and muscle *Arnt*-like protein-1) proteins and their homologs, which dimerize and bind to *cis*-acting E-box elements (with the simple consensus DNA sequence CAANTG) to activate the transcription of a large number of circadian genes. Among these genes are loci encoding the PERIOD and CRYPTOCHROME families of repressor proteins (PER1–3 and CRY1–2), whose products multimerize and suppress the CLOCK:BMAL1 activating complex. Also among the genes activated by CLOCK:BMAL1 is the *Rev-Erba* gene, which encodes a nuclear orphan receptor protein that, together with its sister protein REV-ERB $\beta$ , represses *Bmal1* transcription in a parallel but interlocked loop. The ROR (retinoic acid receptor–related orphan receptor) family of transcriptional activators likely competes with the REV-ERB family of repressors for the same binding sites, adding further cooperativity to the transition mechanism. Numerous reviews have been written about this basic oscillatory circuitry (5).

At each of these steps, additional precision and regulatory finesse are achieved through interaction with a wide range of auxiliary proteins: kinases that phosphorylate clock proteins to modify their stability or activity (6); chromatin-modifying proteins that phosphorylate, acetylate, or deacetylate histones and, in some cases, clock proteins that regulate chromatin structure and



**Figure 1**

The canonical mammalian circadian oscillator and output relevant for xenobiotic metabolism. Two interlocked feedback loops composed of activators (*green*) and repressors (*red*) drive the expression of output genes such as those important for xenobiotic metabolism. Components of these loops make extensive use of auxiliary factors including histone methyltransferases (HMTs), histone deacetylases (HDACs), *Drosophila* behavior/human splicing (DBHS) family RNA-binding proteins (*beige*), and kinases and proteasome machinery (*gray*). Important output genes involved in transcriptional control of the detoxification metabolism are shown in blue. Other abbreviations: ALAS1, aminolevulinic acid synthase 1;  $\beta$ -TrCP,  $\beta$ -transducin repeat-containing protein; CAR, constitutive androstane receptor; Ck1 $\delta/\epsilon$ , casein kinase 1 delta/epsilon; CRY, cryptochrome; Fbx1, F-box and leucine-rich repeat protein; PARbZip, proline and acidic amino acid-rich basic region/leucine zipper protein; PER, period; POR, cytochrome P450 oxidoreductase; ROR, retinoic acid receptor-related orphan receptor; RORE, ROR element.

transcriptional activation potential (7); and RNA-binding proteins that serve as scaffolds for coactivating and corepressing activities (8). This basic clock mechanism is summarized in **Figure 1**. A circadian mechanism independent of transcription also exists in parallel with the canonical transcription-translation-based clock in mammalian cells. Evidence of this oscillator exists in the form of diurnal variation in oxidation states of hemoglobin and antioxidant molecules (9). Both the mechanism and the physiological relevance of these posttranslational clocks remain unknown in mammals, although posttranslational clocks based on phosphorylation are well studied in bacteria (10).

### Hierarchical Organization of Clocks

The basic timekeeping mechanism of circadian oscillators is cell autonomous, and self-sustained clocks exist in most cells of the body. However, under most circumstances, these clocks are organized into a hierarchy: A “master clock” tissue within the suprachiasmatic nuclei (SCN) of the hypothalamus receives light input via the retina and communicates timing signals to

peripheral “slave” oscillators of similar molecular mechanism in cells from other tissues. Multiple redundant signals have been described. These include (a) direct signals such as innervation by the autonomous nervous system and hormones such as glucocorticoids and (b) indirect signals emanating from SCN-controlled rhythmic behavior, such as timing of food intake and small rhythmic changes in body temperature from activity (11).

Under most circumstances, entrainment signals from the SCN to clocks in peripheral tissues act in a concerted fashion, resulting in somewhat coherent phase among different organs. The exact phase of circadian clocks varies somewhat from organ to organ, perhaps because of tissue-specific differences in clock gene expression or because of local differences in accessibility to entrainment signals. These differences become particularly acute under certain perturbation. For example, during an altered lighting cycle caused by time zone travel or shift work, the SCN shift their phase much more quickly (within a day or two) than do peripheral clocks (which can take a week or more), creating a situation in which clocks in different organs exhibit gross differences in “internal clock time” (12). Similarly, systematic manipulation of external cues such as feeding time to different phases of the light-dark cycle result in a phase change for peripheral clocks but not for the SCN clock (13).

This hierarchical clock structure has two important implications for chronopharmacology. First, if clocks in different tissues govern different aspects of drug activity and metabolism—a topic that we explore in detail below—then these different phases must be considered in calculating the timing for optimal drug efficacy. The situation is further complicated because recent research suggests that these phase relationships are altered by age: Older rodents show later SCN phases but earlier peripheral clock phases (14). Second, increasing evidence suggests that chronic circadian dysphasing itself has significant negative consequences for health, both for rodents subjected to laboratory conditions of chronic jetlag or shift work and for humans subjected to similar stresses. Documented changes include cancer susceptibility, inflammation, and altered metabolism (15–17). Thus, increasing evidence suggests that basal physiology may differ in individuals with clock disruption. Such an observation is particularly relevant to pharmacology because many diseases ranging from psychiatric and neurodegenerative disorders to cancer are themselves associated with mild to severe clock disruption (18, 19). The question of how a disease that is specific to a peripheral organ might affect clockwork within that tissue has not been studied, but it could also be highly pertinent to pharmacology.

## Circadian Control of Cellular Physiology

Whereas SCN clocks are entrained by light and peripheral clocks are entrained by indirect and hormonal cues, individual aspects of cellular physiology are, in turn, directed by both local and central clocks through a variety of mechanisms. One fundamental mechanism is via transcription: In total, approximately 10% of all transcripts in each tissue are regulated in a circadian fashion (20, 21). In large part, this regulation occurs through the same *cis*-acting promoter elements that direct the rhythmic transcription of the clock genes themselves, such as the E-boxes that serve as platforms for activation by CLOCK and BMAL1 and the ROR elements that respond to REV-ERB proteins. Regulation of additional genes can occur through cascades of clock-regulated transcription factors. Among the best-studied are the PARbZip (proline and acidic amino acid-rich basic region/leucine zipper protein) family of factors: DBP (D-albumin binding protein), TEF (thyrotrophic embryonic factor), and HLF (hepatic leukemia factor). The D-elements to which they bind control the circadian expression of several families of genes, including liver metabolic regulators critical to circadian control of PK for a wide variety of drugs (22). Modeling studies suggest that simple combinations of these three elements—E-boxes, D-boxes, and ROR elements, each with

maximal occupancy at a certain time of day—can direct circadian transcription in any phase and are responsible for a large portion of circadian transcription directed by cellular clocks (23).

Nevertheless, this mechanism represents only a portion of circadian transcription in living animals. Experiments in mice lacking functional clocks in specific tissues show that only a portion of circadian gene expression is abolished by such manipulations, whereas another portion persists because it is systemically driven (24). A portion of this transcription likely arises through rhythmic activity of the hypothalamic-pituitary-adrenal axis, and another portion arises through circadian stimulation of actin/SRF signaling by unknown ligands (25). Additional contributions likely arise from heat shock signaling and immune signaling, also regulated by time of day. In all four cases, specific externally activated transcription factors bind to *cis*-acting elements to drive the transcription of certain genes. For example, rhythmic glucocorticoid production results in the rhythmic activation of glucocorticoid receptor, which binds to cognate glucocorticoid receptor elements to activate or repress transcription (26). Likewise, circadian body temperature variation results in rhythmic occupancy of heat shock elements (27). The result is circadian transcription of specific genes that is due to cell-extrinsic influences and is independent of the circadian clockwork present in that cell or tissue.

In addition to circadian transcription, recent research has unearthed extensive evidence of circadian posttranscriptional regulation in mammals—including translational control (28); control of transcription termination and/or elongation (29); and, to a lesser extent, circadian control of splicing (30). Thus, the actual number of transcripts showing circadian abundance is significantly greater than the number of genes transcribed in a circadian fashion (31–33), and the number of proteins that are expressed in a circadian fashion is greater than the number of transcripts for which this question has been addressed (34). Major signaling molecules such as cAMP show circadian variations that both control clock output and play a role within the clock (35), and recent links between clocks and sirtuins suggest a similar influence of redox potential (36). Finally, a significant fraction of histone posttranslational modifications vary in a circadian fashion at a large number of loci (31, 32). Altogether, through a myriad of different mechanisms, a significant amount of cellular physiology is regulated by the circadian clock.

One case of such regulation meriting special attention is the circadian regulation of the cell cycle and DNA repair. Given the central importance of cell cycle deregulation in cancer—a disease treated separately below—it is easy to understand why circadian control of cell division in adult animals could be of central importance in clinical pharmacology. In fact, multiple studies have documented circadian or diurnal regulation of cell division, both *in vivo* during rodent liver regeneration (37) and *in vitro* in cultured cells (38). Even in humans, skin blister transcriptional profiling suggests a similar link (39). Moreover, multiple direct connections have been established between the circadian clock and cell cycle checkpoints, including via the checkpoint proteins WEE1 (37), p21-WAF (cyclin-dependent kinase inhibitor 1) (40), and CHK1/2 (checkpoint kinase 1/2) (41) and by control of the circadian transcription of the *p16-Ink4a* locus through the clock-associated NONO protein. In the latter case, this circadian interaction is directly important for tissue regeneration (42). Related to circadian cell cycle control, extensive regulation of DNA damage repair by the circadian clock has also been documented (43), and this control would directly influence susceptibility to cancer.

## FROM CIRCADIAN CONTROL OF PHYSIOLOGY TO CHRONOPHARMACOLOGY

As demonstrated above, at a cellular level, large portions of cellular physiology—from transcription and translation to intracellular signaling cascades—can show daily variations in activity. This

cellular diurnal variation translates directly into diurnal physiological variation in most organ systems, which, in turn, provides the mechanistic rationale for circadian variation in PK/PD.

## Neurotransmitters and Circadian Behavior

Nearly all behaviors show diurnal patterns of activity. In most cases, these oscillations manifest themselves independently of the external environment or the sleep-wake cycle. For example, long-term memory shows a direct dependence on the circadian oscillator: Rodents and humans learn better at certain times of day than others, and mice with functional circadian systems learn better than those without (44). Similarly, anxiety behaviors show a clear diurnal pattern that is modulated both by the sleep-wake cycle and by the circadian oscillator, and these behaviors are elevated in mice lacking the *Period* clock genes (45). Even perception of multiple types of pain varies in a circadian fashion in both humans and animal models (46).

The likely basis for these circadian variations is that virtually all major neurotransmitter systems show either marked circadian variations or clock interactions. For example, circadian variations in opioid receptor abundance, as well as in the abundance of natural opioids themselves, have been reported numerous times over the past two decades (46). The serotonergic system shows clear ultradian variations corresponding to sleep state, but these faster oscillations interact markedly with the circadian clock, and serotonergic signals appear necessary for the integration of circadian information by the basal forebrain in controlling sleep timing (47). In the cholinergic system, numerous circadian variations have also been documented. For example, after a sustained attention task with daily repetition, a daily increase in prefrontal cholinergic neurotransmission is observed even in the absence of the task (48). In general, the cholinergic system plays a critical role in this type of circadian “time-stamping” behavior. This behavior is sustained by the circadian release of acetylcholine during the active phase of many mammals, accompanied by an increase in choline acetyltransferase and a decrease in acetylcholinesterase activity. Globally, the expression of muscarinic acetylcholine receptors shows a pattern inverse to that of acetylcholine availability, with increased abundance during the quiescent phase of the 24-h day, irrespective of activity per se (49). Examination of the dopaminergic system also shows a diurnal pattern of dopamine abundance within the rodent forebrain. Interestingly, this circadian expression appears necessary for the oscillation of the circadian clock gene *Per2* in forebrain neurons, suggesting that dopamine plays a role in mediating circadian information to this brain region (50).

Multiple other neurotransmitters show circadian abundance that strongly interacts with the sleep-wake cycle. For example, adenosine shows circadian variations within the brain that are believed to be sleep-wake dependent (51). More broadly, purinergic signaling shows a strong circadian component and interacts directly with the circadian machinery through ATP, cAMP, and AMP (52). The hypocretin/orexin system also has circadian variation that regulates REM sleep in particular (53). Circadian release of  $\gamma$ -aminobutyric acid (GABA) and glutamate—the principal inhibitory and excitatory neurotransmitters of the brain, respectively—controls not only behavior but also hypothalamic hormone release, which regulates many aspects of physiology (54).

## Circadian Hormones, Cellular Clocks, and the Control of Metabolism, Digestion, and Cardiac Function

Beyond the neurotransmitters whose circadian output is directly or indirectly regulated by the SCN, numerous other hormones show diurnal regulation that significantly regulates physiology and pharmacology. Melatonin, a circadian hormone of the pineal gland, influences various aspects



of retinal (55) and cardiovascular (56) function and affects local clocks in diverse brain regions (57). Circadian regulation of the adrenal gland results in diurnal secretion of glucocorticoid hormone, which, in turn, strongly influences metabolism and in fact directly regulates 60% of the liver transcriptome (58). Circadian regulation of gastrin, ghrelin, and somatostatin, as well as direct regulation by autonomous clocks within the gastrointestinal tract, mediate circadian influences on digestive function (59).

More generally, autonomous circadian clocks not only within the gastrointestinal tract but also in numerous other tissues have considerable influences on physiology and metabolism. For example, ablation of clocks in pancreatic islets results in diabetes because of defects in the coupling of  $\beta$  cell stimulus to insulin secretion (60), and local clockwork controls the expression of multiple ion channels and kinases in heart that influence cardiac function and triglyceride metabolism (61, 62). Recent transcriptome studies have identified widespread local circadian regulation not only in heart but also in skeletal muscle and fat, showing that clocks in these tissues directly regulate physiology (63).

### **Circadian Immune Regulation**

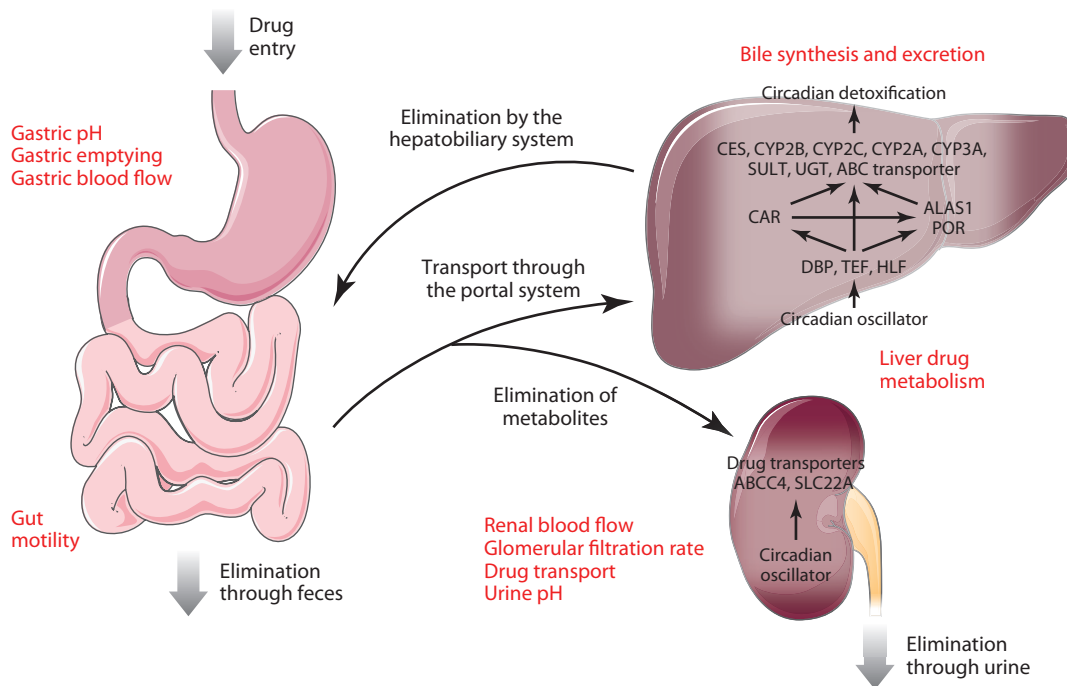
A second prominent pharmacological target with strong circadian regulation is the immune system. Diurnal variations in white blood cell count and susceptibility to endotoxic shock have long been documented. However, recent research shows that cell-autonomous clocks within immune cells themselves direct variation in a large number of circadian immune parameters. For example, the response of T cells to stimulation varies in a circadian fashion (64), and macrophages, in turn, stimulate immune responses in an equally diurnal fashion with their own clocks (65). By contrast, far fewer reports of circadian B cell activity exist, and, indeed, the oscillations documented in circadian gene expression in peripheral blood mononuclear cells are mostly much lower in amplitude than those observed in other tissues such as the liver.

The consequences of pervasive circadian regulation of immune function are numerous and range far beyond the aforementioned diurnal variation in infective susceptibility. For example, a pronounced circadian oscillation of blood clotting has long been known and is supported by circadian variation in factors ranging from platelet aggregation and adhesion (66) to the actual expression of clotting factors such as PAI-1 (plasminogen activator inhibitor-1) (67). Circadian clocks also regulate circulation of many immune cells such as hematopoietic stem cells (68). Finally, circadian immune regulation results in diurnal variations in related immune parameters such as inflammation, which plays a strong role in circadian variation in many diseases (69).

## **CIRCADIAN PHARMACOKINETICS: OSCILLATIONS IN JEJUNAL, HEPATIC, AND RENAL SYSTEMS**

### **Rhythmic Gastric and Intestinal Absorption**

Drug transport and diffusion are highly dependent on gastric pH, which regulates drug ionization and hydrophobicity. In most animal species including humans (70), gastric pH presents a strong circadian pattern influencing drug solubility. At the same time, gastric emptying after a meal (71) and gastrointestinal mobility (72) occur at higher speed during the day than at night, thus increasing absorption during the day. Interestingly, at least for the colon, this rhythmic motility seems to be regulated by the circadian clock, as it is severely perturbed in mice without a clock (73). Finally, the increased blood flow to the gastrointestinal tract at the beginning of the day also contributes to increased drug distribution in daytime in humans (74) (**Figure 2**).



**Figure 2**

Modulation of drug pharmacokinetics by the circadian clock. All rhythmic parameters influencing drug transport and metabolism are highlighted in red. Characteristic functions of the circadian clock in these processes are indicated in the respective organs.

Abbreviations: ABC, ATP-binding cassette; ALAS, aminolevulinic acid synthase; CAR, constitutive androstane receptor; CYP, cytochrome P450; DBP, D-albumin binding protein; HLF, hepatic leukemia factor; POR, P450 oxidoreductase; SLC, solute carrier family; SULT, sulfotransferase; TEF, thyrotrophic embryonic factor; UGT, UDP-glucuronosyltransferase.

### Rhythmic Liver Drug Metabolism

Xenobiotic detoxification is organized as a multistep system consisting of three groups of proteins assuming distinct and successive functions (75). The phase I proteins functionalize drugs (possibly for inhibition or activation) by the oxidase, reductase, and hydroxylase activities of the microsomal cytochrome P450 (CYP P450) family of enzymes. The phase II proteins conjugate drugs to a hydrophilic molecule to increase solubility. They help make lipophilic compounds hydrophilic enough to facilitate their excretion into urine, bile, and feces. These reactions are achieved mainly by sulfotransferases, UDP-glucuronosyltransferases, glutathione S-transferases, and N-acetyltransferases. Finally, phase III transporters—mainly ATP-binding cassette (ABC) transporters—transport xenobiotics outside the cell. Inversely, transporters of the solute carrier family (SLC) are involved in cellular import.

In addition to these three classes of enzymes, other proteins globally regulate the activity of most of the phase I enzymes of the cytochrome P450 family. For example, aminolevulinic acid synthase 1 (ALAS1) is the rate-limiting enzyme in the synthesis of heme, the prosthetic group of all CYP P450 enzymes, and is therefore required for CYP P450 synthesis (76). Moreover, the monooxygenase reaction catalyzed by CYP P450 enzymes requires electrons that are extracted from NAD(P)H and transferred via the flavin group of the CYP P450 oxidoreductase (POR) enzyme to the heme group (77).



The expression of all of these proteins is carefully coordinated to favor efficient liver detoxification, and this coordination is important for PK. This control is achieved through the complex transcriptional regulation of these genes in a manner that is cell type specific, daytime dependent, and inducible by xenobiotics themselves. Transcriptional induction involves a heterogeneous class of transcription factors collectively named xenobiotic receptors. The three main xenobiotic receptors are the nuclear receptors constitutive androstane receptor (CAR) and pregnane X receptor (PXR) (78) and the Per-Arnt-Sim (PAS)-domain helix-loop-helix transcription factor aryl hydrocarbon receptor (AhR) (79). Mainly expressed in the liver and the small intestine, these xenobiotic receptors are associated with chaperone proteins in the cytoplasm. In response to xenobiotics—either through direct binding or by way of signal transduction—these proteins accumulate in the nucleus, where they activate the transcription of phase I, II, and III genes.

Historical transcriptome analysis of mouse liver revealed that genes coding for enzymes involved in the three phases of xenobiotic detoxification represent an important part of the rhythmically expressed genes (20). Recent evidence suggests that these genes are not direct targets of BMAL1; rather, it suggests that they are regulated by circadian clock-controlled transcription factors (80). Studies in genetically engineered mice revealed the pivotal role of the PARbZip transcription factors in achieving this regulation. Their importance is highlighted by deletion studies: Mice devoid of these three genes are born with a normal Mendelian ratio and no apparent phenotypes, but fewer than 20% of them are still alive after one year (81).

Gene expression analysis revealed that PARbZip-deficient mice show a general decrease in the expression of genes coding for enzymes involved in xenobiotic detoxification of all phases in liver and kidney (22). PARbZip proteins probably regulate the expression of some of these enzymes through rhythmic binding to their promoters, for example *Cyp3a4* (82) and *Mdr1a* (*multidrug resistance 1a*; also known as *Abcb1a*) (83). However, another important mode of activity also exists: PARbZip transcription factors also regulate the expression of CAR (22), which is strongly decreased with a dampened rhythm in the liver and small intestine of PARbZip-deficient mice. As a consequence, induction of phase I, II, and III enzymes is very low throughout the day in these animals. Thus, the time-dependent induction of *Cyp2b10* mRNA by phenobarbital is strictly compromised in PARbZip-deficient mice. These mice are also susceptible to toxicity from cyclophosphamide and mitoxantrone, two drugs used for the chemotherapeutic treatment of cancer. This phenotype is shared by mice deficient in the clock genes *Clock* and *Bmal1* themselves, because these mice also display a low level of expression of PARbZip transcription factors (R. Dallmann, J.P. Debruyne & D.R. Weaver, submitted manuscript). Interestingly, the time-dependent toxicity of pesticide in *Drosophila* involves similar mechanisms, through the regulation of the CAR ortholog DHR96 by the PARbZip ortholog *Pdp1* (84) and the rhythmic expression of phase II enzymes (85).

Despite the importance of the PARbZip transcription factors in circadian PK, other clock-regulated genes are also involved in rhythmic drug detoxification. For example, ROR $\alpha$  and ROR $\gamma$  knockout mice display a deficiency in expression of several phase I and phase II enzymes, probably also as a result of perturbed expression of the CAR xenobiotic receptor (86). In addition, mRNA coding for the other xenobiotic receptors *Pxr* (87) and *Abr*, along with *Abr*'s heterodimerization partner *Arnt* (88), results in a rhythmic pattern of expression. In the latter case, AhR's main target *Cyp1a1* is not only rhythmically expressed (88) but also induced in a time-dependent manner by the AhR ligand 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (89). This time-dependent induction of *Cyp1a1* requires a functional circadian clock (90). Finally, acetaminophen time-dependent toxicity seems to be a result of the rhythmic expression of CYP2E1 (91), due itself to the rhythmic inhibition of HNF1 (hepatocyte nuclear factor 1) by CRY1 on the *Cyp2e1* promoter (92). Whereas the relative importance of all these systems in global rhythmic drug detoxification in mouse liver has not been

clearly demonstrated, there is no doubt that the circadian clock is a major actor in this arena (Figure 2).

### Rhythmic Elimination by the Hepatobiliary System

Although most metabolized drugs are finally excreted into plasma and subsequently urine, several of them are first excreted through the hepatobiliary system into the gut and are subjected to a second round of hepatic metabolism or fecal excretion. The hepatobiliary transport system is required not only for bile formation but also for elimination of various endo- and xenobiotics including cholesterol, phospholipids, and drugs (93). Depending on the nature of the molecule, a broad range of liver-specific export systems are involved. Bile is formed by the excretion of bile salts (BS) and nonbile salt organic anions via ABC transporters. Monovalent BS are excreted via the bile salt export pump (BSEP or ABCB11), whereas divalent BS and anionic conjugates of endo- or xenobiotics are excreted via the conjugate export pump (MRP2 or ABCC2). The phospholipid export channel (MDR2 or ABCB4) allows the excretion of phosphatidylcholine, which forms micelles in bile together with BS and cholesterol. Cationic metabolized drugs are excreted by the multidrug export channel (MDR1 or ABCB1). Other export pumps include the two-half transporter ABCG5/8 for cholesterol and the breast cancer resistance protein (BCRP or ABCG2) for anionic conjugates.

The excretion of bile acids, lipids, and xenobiotics into the bile follows a stringent circadian rhythm, at least in rodents (94), and clock involvement has been documented at multiple steps. First, bile acid synthesis follows a stringent diurnal rhythm in both rodent (95) and human (96). Conversion of cholesterol into bile acids involved the rate-limiting cholesterol-7 $\alpha$ -hydroxylase (CYP7A1), whose rhythmic expression is directly regulated mainly through REV-ERB $\alpha$  (97–99). In addition, most of the genes encoding transporters involved in bile secretion are expressed according to a circadian pattern in the liver, even if the mechanism has not been clearly described (20). As a consequence, the biliary excretion of drugs, for example ampicillin (100) and flomoxef, exhibits a diurnal pattern in rats (101) and patients under percutaneous transhepatic biliary drainage (102) (Figure 2).

### Rhythmic Elimination by the Kidney

Most water-soluble drugs or drug metabolites are eliminated by urine through the kidney. The rate of drug elimination in the urine depends on several intrinsic variables related to kidney function including the renal blood flow (RBF); the glomerular filtration rate (GFR); the capacity of the kidney to reabsorb or to secrete drugs across the epithelium; the urine flow; and the urine pH, which influences the degree of urine acidification. Interestingly, all these variables show a circadian pattern in different mammalian models.

Approximately 20% of the blood from the RBF is converted into primary urine through glomerular filtration. In the proximal tubule, many ionized drugs can be secreted in the urine from the remaining unfiltered blood via various active transports involving SLC and ABC transporters. Finally, filtered and secreted drugs can be passively or actively reabsorbed out of the urine into the blood. Because RBF is a key determinant of glomerular filtration and secretion, it is intimately associated with the elimination of most ionized drugs through urine. RBF follows a circadian oscillation with a peak during the active phase (103). Although this rhythm is probably partially entrained by circadian arterial blood pressure and cardiac output, the rhythmic RBF could also be generated by an intrinsic renal mechanism. For example, *Cry1/Cry2* knockout (104) results

in disrupted activity of the renin-angiotensin-aldosterone system, one of the major mechanisms regulating RBF.

Rhythmic oscillations of the GFR are synchronized with those of RBF, but they are not fully determined by it, as GFR rhythm persists during continuous bed rest and in the condition of inverted blood pressure (103). Rhythmic GFR is also maintained in transplanted human kidneys, indicating that sympathetic innervation is not required for this rhythm (105). These data indicate that GFR is generated by an intrinsic renal mechanism, but the mechanism responsible for this functional rhythmicity remains unknown.

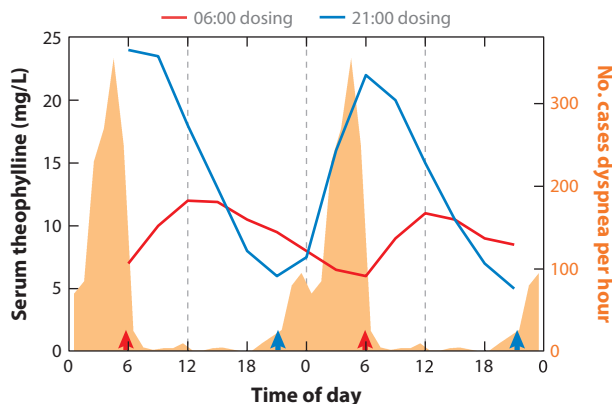
Renal reabsorption and secretion of water-soluble drugs depends on the expression of membrane transporters of the ABC and SLC families that facilitate diffusion of polar molecules through the apical and/or basolateral membranes of tubular cells. Most of drug reabsorption/secretion takes place in the proximal tubule of the kidney, which is enriched in various transporters with a preferential affinity for small organic anions (106). Several of these transporters exhibit robust diurnal expression in the more distal nephron segments, namely in the distal nephron and the collecting duct (107). Moreover, the expression of MRP4 (ABCC4) and that of OAT2 (SLC22A7) are significantly reduced in the kidney of PARbZip knockout mice, providing direct evidence for circadian clock-controlled tubular reabsorption/secretion (22).

Drug ionization, which is mainly determined by urine pH, determines drug solubility and the rate of drug reabsorption in the nephron. Human urine pH may range from 4.5 to 8 and is controlled by a complex system of reabsorption/secretion/production of bicarbonate and secretion of protons. It usually exhibits lower values in the morning. The most important transporter involved in renal proton secretion is the sodium-proton exchanger 3 (NHE3 or SLC9A3) expressed in the proximal tubule. The expression of *Nhe3* mRNA and protein in the kidney causes a robust circadian rhythm in rodent, with maximal expression in the middle of the active phase (108). Interestingly, this circadian expression is significantly blunted in *Cry1/Cry2* knockout mice, indicating that the circadian clock can influence the renal drug disposal via the control of urine acidification (Figure 2).

## CHRONOBIOLOGICAL IMPLICATIONS FOR DRUG TREATMENT

To what extent has the knowledge presented above translated to effective pharmaceutical interventions? The most obvious examples of successful chronotherapy are ones with obvious time-of-day-dependent symptoms. Treatment of bronchial asthma has been tuned to result in maximum plasma levels when dyspnea most frequently occur and therefore to alleviate symptoms most effectively (Figure 3). Similarly, blood pressure shows a sharp peak in the early morning, coinciding with the peak for cardiovascular events (109), and an extended trough during the night. Both healthy normotensive patients and those suffering from essential hypertension exhibit this variation (110). The L-type calcium channel blocker verapamil, for example, uses an extended-release formulation to result in therapeutically effective plasma levels in the early morning, after bedtime oral administration (111). In addition, such delayed-release drugs have been beneficial for hypertensive patients who do not show a nocturnal dip in blood pressure, so-called nondippers (112). Nondipping is a risk factor for congestive heart failure even in clinically normotensive subjects (113, 114).

As mentioned above, not only are PK/PD parameters modulated by time of day, but drug metabolism is as well. For example, over-the-counter acetaminophen [analgesic *N*-acetyl-*p*-aminophenol (APAP)] is a leading cause of drug-induced liver failure in the United States (115). APAP toxicity is dependent on generation of *N*-acetyl-*p*-benzoquinone imine (NAPQI) by the CYP P450 system of the liver (116), mainly CYP2E1 (92). APAP toxicity is time-of-day



**Figure 3**

Time-of-day-dependent variation in pharmacokinetics. Average 3-hourly steady-state serum concentrations of theophylline from eight asthmatic children dosed with Theo24<sup>TM</sup> before breakfast (06:00, *red*) or at bedtime (21:00, *blue*), as indicated by arrows. Shaded area represents the occurrences of dyspnea. Data from References 165 and 166.

dependent (3, 91), but liver-specific ablation of the clock in mice blunts this rhythm (R. Dallmann, J.P. Debruyne & D.R. Weaver, submitted manuscript).

## Cancer

Whereas the chronotherapeutic approach of the examples above is based on relatively few well-established variables, in the case of chemotherapy and associated cancer treatments, the predictions for optimal treatment schedules become highly complex. On one hand, chemotherapeutics should be dosed high enough to be toxic to the cancer; on the other hand, the dose should be low enough to prevent serious damage to healthy tissue or organs. That means PK/PD operate in a tight therapeutic range. Under these preconditions, the variations introduced by the circadian system on multiple levels can be crucial. This is further complicated by the possibility not only that the healthy tissue has a clock but also that the tumor has one. *In vivo*, this has been shown by measuring the incorporation of P<sup>32</sup> in tumors of terminally ill breast cancer patients (117). These results are in line with newer *in vitro* data from various human and mouse cancer cell lines such as the human U2 osteosarcoma (U2OS) line (118). A clock in the tumor is an important factor because most cancer drugs are toxic only to dividing cells or have a mechanism of action that is particularly effective in one phase of the cell cycle, which is—at least in healthy tissues—gated by the clock. The topoisomerase I inhibitor irinotecan, for example, is most effective in S-phase, whereas alkylating agents cross-link DNA at any phase of the cell cycle (119). In the case of an arrhythmic tumor, as in the mouse xenograft Glasgow osteosarcoma model, a further interesting complication emerged: Seliciclib, a cyclin-dependent kinase inhibitor, seemed to induce rhythmic gene expression in tumors, a process that might slow down tumor progression (120). Given the known disturbance of circadian behavior in multiple human cancers, additional efficacy might be achieved harmlessly by this type of clock resynchronization.

Overall, in several different experimental rodent models, efficacy and side effects of anticancer therapies vary up to 10-fold depending on time of day. However, these parameters are model and drug specific. Common to most therapies is that efficacy is based on the mechanism of action, metabolism, and toxicity, and the best treatment schedule has to take into account all those

parameters. The therapeutic index of the alkylating agent cyclophosphamide, for example, is significantly better when the compound is administered during the first part of the active dark phase (121). This rhythm may be dependent on CLOCK:BMAL1 binding in B cells (121); changes in CYP P450 enzyme activity; and, even more important, higher reduced glutathione levels at night, as has been described for other alkylating agents such as cisplatin. In contrast, 5-fluorouracil's (5-FU's) first and rate-limiting step in metabolism is dependent on the availability of dihydropyrimidine dehydrogenase (DPD), and certain 5-FU metabolites then block the activity and de novo synthesis of thymidylate synthase (TS), which is important for DNA synthesis (122). Expression of DPD and that of TS are high and low, respectively, during the first part of the light phase. Therefore, 5-FU exhibits best tolerability and efficacy 180° out of phase with cyclophosphamide and most other alkylating agents. Leucovorin is an inhibitor of TS and often coadministered with 5-FU. It adds to the effectiveness of 5-FU and changes the DPD-to-TS ratio in the same direction, as observed at the optimal time of day established in animal experiments.

Interestingly, irinotecan and 5-FU have even more traits in common. For both, added value of chronotherapeutic treatment regimens is gender specific in experimental animal models. In the case of 5-FU, this gender specificity has been observed even in clinical populations (123). Whereas chronomodulated delivery of 5-FU improves survival for male patients compared with conventional treatment, the opposite was true for female study participants, as found in a study by Innominato and colleagues (124). The authors speculate that because disruption of the rest/activity rhythm during chemotherapy predicts overall survival for metastatic colorectal cancer (124), the men could exhibit more robust circadian rhythms. However, further investigations are needed.

## Implications for Drug Discovery and Development

Classically, the drug discovery process is preceded by the validation of a given target. The mechanism of action is established and molecular targets defined. Taking diurnal changes of relevant parameters into account might mean significantly higher costs because the same experiments might have to be conducted at multiple times of day in order to assess if, for example, a certain type of receptor or protein is expressed only at a specific time of day. However, online resources can be mined for information about the circadian expression of a given transcript or metabolite (125, 126). A special case involves the quest for drugs against aging-related diseases. As do human beings, rodent species typically used in these assays exhibit attenuated circadian rhythms. Thus, the PK/PD profile and target availability could change during the course of aging.

Once the target is confirmed and the lead optimization process has started, the properties of the novel chemical entities are evaluated and selected. Typically, CYP P450 induction and inhibition in human and rodent primary hepatocytes are tested. This testing might introduce bias toward only one phase of the circadian cycle because the cells that are used to evaluate the compounds contain a functional cell-autonomous clock that can influence drug metabolism, as detailed above. The CACO-2 monolayer assay is an industry standard used not only to predict absorption after oral application through the intestinal barrier but also to assess interactions with important transporters such as P-glycoprotein (127). Interestingly, like the intestinal barrier itself, the human tumor-derived CACO-2 cells have a functioning clock (128) that directly controls expression levels of *Mdr1* (83).

As mentioned above, two of the most common reasons for novel chemical entities to fail in drug development or even for marketed drugs to be withdrawn are liver toxicity and cardiac safety. In fact, QT prolongation often associated with blockade of the K<sup>+</sup> channel encoded by human ether-à-go-go related gene (hERG) and a surrogate marker for torsade des pointes (129)

was the single most common cause for withdrawal of marketed drugs in recent years (see 130 and <http://www.ich.org/>). Therefore, an extensive battery of tests ranging from in vitro channel function to in vivo electrophysiology in the freely moving dog or monkey has been established (131); these tests are performed before a so-called thorough QT/QTc (132) study in Phase I of development. Although new models adjust for circadian variability due to changes in heart rate over the day (133, 134), the possibility that drug-dependent QT-interval prolongation is directly influenced by time of day in patients has not been fully explored but is not unsupported (135). Moreover, there is a clear rationale for how the circadian clock would influence cardiac repolarization, namely via *Krüppel-like factor 15* (136). Together, these findings suggest that a time-of-day bias in testing drug-induced QT prolongation might lead to a misjudgment of risk to patients.

If, on the basis of these considerations, drug developers were to adopt a circadian testing policy, a further complication would need to be addressed. In contrast to the circadian phenotypes in most preclinical animal models, those in people exhibit a great deal of interindividual variability: The period or phase and amplitude of the clock-controlled rhythms described above varies greatly in human populations (137). These are no small differences, and the lay terms larks and owls to describe people with early and late activity phases, respectively, illustrate their magnitude. Moreover, diseases can even more severely alter rhythmic rest/activity and endocrine patterns. Some schizophrenic patients, for example, exhibit a nearly arrhythmic behavioral pattern (138); in depressed patients, cortisol levels are elevated, but their diurnal variation is blunted (139). Twin studies suggest that genetic traits partially determine the chronotype (140). In fact, multiple loci contribute to differences in chronotype and sleep (141). Moreover, there are quite consistent age-dependent changes of chronotype (142), and recent results suggest relatively stable changes dependent on previous light history (143).

Interindividual differences in clock phase are sizable and therefore probably clinically relevant. In theory, the properties of an individual's circadian clock can be determined in a simple test, but in practice, such a determination has not been possible so far. The ability to use human skin biopsies that are lentivirally transduced with circadian reporters to determine period is one cellular method that can be used toward this end (144), and hair follicle samples have also been used (145). Alternatively, clock-controlled neuroendocrine signals such as the dim-light melatonin onset have been used to estimate clock phase (146). Due to large interindividual variation in the levels of these hormones, which are also dependent on ambient light levels, multiple sampling is necessary to establish meaningful results. To overcome these limitations, transcriptome and metabolome data sets have been used to establish timetable methods for "internal time" in mice and men (147–149). All of these methods, however, rely on at least two sampling times that are optimally 12 h apart to compensate for interindividual differences in the absolute levels of gene or metabolite expression, and internal time can be approximated with a precision of 2 h. Thus, a feasible and accurate circadian test remains to be established.

Given the aforementioned examples of arrhythmicity in disease and interindividual differences in clock phase, the circadian clock itself might be an interesting target for drug development. In fact, multiple pharmacological agents can phase-shift behavioral and biochemical rhythms in experimental animal models and people; for example, melatonin, melatonin agonists, and a combined melatonin agonist/5-HT<sub>2c</sub> antagonist have already received market approval, although not necessarily as phase-shifting drugs. In addition to the melatonin receptors, several independent molecular targets can alter clock function. Among the first to be identified was *casein kinase 1*, and even subform-specific tool compounds have been described (150). As a target, however, kinase inhibitors are not without problems. *Casein kinase 1*, for example, is involved in *Wnt* signaling and linked to cell proliferation and survival (151).



Furthermore, numerous compounds that target core molecular clock components or are believed to be important links between the clock and hypothalamic-pituitary-adrenal axis have been identified and tested, including a neuropeptide Y Y5 receptor (152), a tachykinin antagonist (153), an inverse agonist of ROR $\alpha$  (154), and a corticotropin-releasing factor antagonist (155). Most recently, several unbiased small-molecule screens have been undertaken in an *in vitro* model of the circadian oscillator, and they have identified new molecular entities that target known clock components such as CRY, CK1, and REV-ERB $\alpha$  (156–158). Whether these *in vitro* data from U2OS cells will be able to translate *in vivo* will be interesting.

More generally, whether the clock does indeed present a druggable target or whether there are unsuspected mechanistic problems in altering circadian rhythms remains to be seen. Should such compounds exhibit safety profiles as favorable as that of melatonin, for example, they could prove useful in a wide spectrum of possible indications, ranging from sleep-wake problems in shift workers to amplitude-related problems in aging-related diseases. On one hand, such drugs could help boost circadian rhythmicity beyond what can be achieved through behavioral measures; on the other hand, they could help readjust specific rhythmic components to a favorable phase. Doing so would be especially useful given the tendency of modern society toward a 24/7 lifestyle.

In this respect, we have seen the accumulation of more and more evidence showing the sometimes disastrous effects of such clock disruption on health (15, 16, 18, 19, 159). Unfortunately, there seems to be a vicious cycle between cause and effect. Clock disruption over time can lead to various major pathologies, and these, in turn, can feed back onto the clock and further abrogate rhythms. Interestingly, however, strengthening the clock by imposing strong timing cues can alleviate symptoms. In a mouse model of Huntington's disease, for example, the use of hypnotics and scheduled meals can normalize circadian gene expression rhythms and improve disease symptoms (160). Similarly, melatonin and bright light treatment have a positive effect on institutionalized patients with Alzheimer's disease (161).

## CONCLUSIONS

The recent findings that we have highlighted yield insight into the growing field of chronopharmacology and into the mechanistic basis for the variations in PK/PD that have been observed in a vast number of instances. However, many important questions remain unanswered. Most if not all of the circadian expression data at the genomic level on which these conclusions are based are available only for rodents. Considering the fact that the expression and functional properties of drug-metabolizing enzymes and drug transporters are highly species specific (162), extrapolation of these results to humans is not a foregone conclusion. To translate research data to clinical application, significant progress in the characterization of circadian variations in protein expression and activity in humans is absolutely necessary.

Although there has been much more awareness of the impact of the circadian clock on health, disease, and treatment in recent years, these findings have not translated to clinics or regulatory agencies on a broad scale. Entering the search term circadian on the ClinicalTrials.gov website yields a list of 205 related clinical trials. Twelve of these trials are cancer related, but none try to establish chronotherapeutic treatment regimens. The search term chronotherapy results in 14 hits. In contrast, the search term cancer produces 38,331 results. Similar results were obtained from the EU clinical trials register. Given the fact that approximately 20% of the transcriptome, proteome, and metabolome are under clock control (34, 163, 164), these results seem disproportionate. In the case of regulatory authorities, none of the chronobiological effects on PK/PD outlined here are mentioned in the guidelines published by the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH). This is

surprising, especially considering that unexpected hepatotoxicity and cardiac side effects are the most common reasons for the withdrawal of marketed drugs.

Finally, the large proportion of physiology regulated by the circadian clock suggests that the clock itself might serve as a possible pharmaceutical target to increase efficacy and reduce side effects of existing drugs. For such treatments to be effective, more detailed knowledge will be required—not only of how clocks control physiology but also of how clocks in different organ systems contribute to different processes relevant to PK/PD.

## DISCLOSURE STATEMENT

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# Contents

|   |     |
|---|-----|
| Learning to Program the Liver<br><i>Curtis D. Klaassen</i> .....  | 1   |
| The Druggable Genome: Evaluation of Drug Targets in Clinical<br>Trials Suggests Major Shifts in Molecular Class and Indication<br><i>Mathias Rask-Andersen, Surendar Masuram, and Helgi B. Schiöth</i> .....  | 9   |
| Engineered Botulinum Neurotoxins as New Therapeutics<br><i>Geoffrey Masuyer, John A. Chaddock, Keith A. Foster, and K. Ravi Acharya</i> .....   | 27  |
| Pharmacometrics in Pregnancy: An Unmet Need<br><i>Alice Ban Ke, Amin Rostami-Hodjegan, Ping Zhao, and Jashvant D. Unadkat</i> .....   | 53  |
| Antiparasitic Chemotherapy: From Genomes to Mechanisms<br><i>David Horn and Manoj T. Duraisingh</i> .....   | 71  |
| Targeting Multidrug Resistance Protein 1 (MRP1, <i>ABCC1</i> ):<br>Past, Present, and Future<br><i>Susan P.C. Cole</i> .....  | 95  |
| Glutamate Receptor Antagonists as Fast-Acting Therapeutic<br>Alternatives for the Treatment of Depression: Ketamine<br>and Other Compounds<br><i>Mark J. Niciu, Ioline D. Henter, David A. Luckenbaugh, Carlos A. Zarate Jr.,<br/>and Dennis S. Charney</i> ..... | 119 |
| Environmental Toxins and Parkinson's Disease<br><i>Samuel M. Goldman</i> .....  | 141 |
| Drugs for Allosteric Sites on Receptors<br><i>Cody J. Wenthur, Patrick R. Gentry, Thomas P. Mathews, and Craig W. Lindsley</i> ..   | 165 |
| microRNA Therapeutics in Cardiovascular Disease Models<br><i>Seema Dangwal and Thomas Thum</i> .....  | 185 |
| Nanocarriers for Vascular Delivery of Anti-Inflammatory Agents<br><i>Melissa D. Howard, Elizabeth D. Hood, Blaine Zern, Vladimir V. Shuvaev,<br/>Tilo Grosser, and Vladimir R. Muzykantov</i> .....   | 205 |

|   |     |
|---|-----|
| G Protein–Coupled Receptors Revisited: Therapeutic Applications<br>Inspired by Synthetic Biology<br><i>Boon Chin Heng, Dominique Auel, and Martin Fussenegger</i> .....                               | 227 |
| Cause and Consequence of Cancer/Testis Antigen Activation<br>in Cancer<br><i>Angelique W. Whitehurst</i> .....  | 251 |
| Targeting PCSK9 for Hypercholesterolemia<br><i>Giuseppe Danilo Norata, Gianpaolo Tibolla, and Alberico Luigi Catapano</i> .....   | 273 |
| Fetal and Perinatal Exposure to Drugs and Chemicals:<br>Novel Biomarkers of Risk<br><i>Fatma Etwel, Janine R. Hutson, Parvaz Madadi, Joey Gareri, and Gideon Koren</i> ...                            | 295 |
| Sodium Channels, Inherited Epilepsy, and Antiepileptic Drugs<br><i>William A. Catterall</i> .....   | 317 |
| Chronopharmacology: New Insights and Therapeutic Implications<br><i>Robert Dallmann, Steven A. Brown, and Frédéric Gachon</i> .....   | 339 |
| Small-Molecule Allosteric Activators of Sirtuins<br><i>David A. Sinclair and Leonard Guarente</i> .....   | 363 |
| Emerging Therapeutics for Alzheimer’s Disease<br><i>Karen Chiang and Edward H. Koo</i> .....  | 381 |
| Free Fatty Acid (FFA) and Hydroxy Carboxylic Acid (HCA) Receptors<br><i>Stefan Offermanns</i> .....   | 407 |
| Targeting Protein-Protein Interaction by Small Molecules<br><i>Lingyan Jin, Weiru Wang, and Guowei Fang</i> .....   | 435 |
| Systems Approach to Neurodegenerative Disease Biomarker Discovery<br><i>Christopher Lausted, Inyoul Lee, Yong Zhou, Shizhen Qin, Jaeyun Sung,<br/>Nathan D. Price, Leroy Hood, and Kai Wang</i> ..... | 457 |
| GABA <sub>A</sub> Receptor Subtypes: Therapeutic Potential in Down<br>Syndrome, Affective Disorders, Schizophrenia, and Autism<br><i>Uwe Rudolph and Hanns Möbler</i> .....                           | 483 |
| Role of Hepatic Efflux Transporters in Regulating Systemic and<br>Hepatocyte Exposure to Xenobiotics<br><i>Nathan D. Pfeifer, Rhiannon N. Hardwick, and Kim L.R. Brouwer</i> .....                    | 509 |
| Turning Off AKT: PHLPP as a Drug Target<br><i>Alexandra C. Newton and Lloyd C. Trotman</i> .....  | 537 |
| Understanding and Modulating Mammalian-Microbial<br>Communication for Improved Human Health<br><i>Sridhar Mani, Urs A. Boelsterli, and Matthew R. Redinbo</i> .....                                   | 559 |

Pharmaceutical and Toxicological Properties of Engineered  
Nanomaterials for Drug Delivery  
*Matthew Palombo, Manjeet Deshmukh, Daniel Myers, Jieming Gao, Zoltan Szekeley,  
and Patrick J. Sinko* ..... 581

**Indexes**

Cumulative Index of Contributing Authors, Volumes 50–54 ..... 599  
Cumulative Index of Article Titles, Volumes 50–54 ..... 602

**Errata**

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#### TABLE OF CONTENTS:

- *What Is Statistics?* Stephen E. Fienberg
- *A Systematic Statistical Approach to Evaluating Evidence from Observational Studies*, David Madigan, Paul E. Stang, Jesse A. Berlin, Martijn Schuemie, J. Marc Overhage, Marc A. Suchard, Bill Dumouchel, Abraham G. Hartzema, Patrick B. Ryan
- *The Role of Statistics in the Discovery of a Higgs Boson*, David A. van Dyk
- *Brain Imaging Analysis*, F. DuBois Bowman
- *Statistics and Climate*, Peter Guttorp
- *Climate Simulators and Climate Projections*, Jonathan Rougier, Michael Goldstein
- *Probabilistic Forecasting*, Tilmann Gneiting, Matthias Katzfuss
- *Bayesian Computational Tools*, Christian P. Robert
- *Bayesian Computation Via Markov Chain Monte Carlo*, Radu V. Craiu, Jeffrey S. Rosenthal
- *Build, Compute, Critique, Repeat: Data Analysis with Latent Variable Models*, David M. Blei
- *Structured Regularizers for High-Dimensional Problems: Statistical and Computational Issues*, Martin J. Wainwright
- *High-Dimensional Statistics with a View Toward Applications in Biology*, Peter Bühlmann, Markus Kalisch, Lukas Meier
- *Next-Generation Statistical Genetics: Modeling, Penalization, and Optimization in High-Dimensional Data*, Kenneth Lange, Jeanette C. Papp, Janet S. Sinsheimer, Eric M. Sobel
- *Breaking Bad: Two Decades of Life-Course Data Analysis in Criminology, Developmental Psychology, and Beyond*, Elena A. Erosheva, Ross L. Matsueda, Donatello Telesca
- *Event History Analysis*, Niels Keiding
- *Statistical Evaluation of Forensic DNA Profile Evidence*, Christopher D. Steele, David J. Balding
- *Using League Table Rankings in Public Policy Formation: Statistical Issues*, Harvey Goldstein
- *Statistical Ecology*, Ruth King
- *Estimating the Number of Species in Microbial Diversity Studies*, John Bunge, Amy Willis, Fiona Walsh
- *Dynamic Treatment Regimes*, Bibhas Chakraborty, Susan A. Murphy
- *Statistics and Related Topics in Single-Molecule Biophysics*, Hong Qian, S.C. Kou
- *Statistics and Quantitative Risk Management for Banking and Insurance*, Paul Embrechts, Marius Hofert

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