

Published in final edited form as:

Annu Rev Pharmacol Toxicol. 2014 January 6; 54: . doi:10.1146/annurev-pharmtox-011613-135923.

Chronopharmacology: New Insights and Therapeutic Implications

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1 Abstract

Most facets of mammalian physiology and behavior vary according to time-of-day thanks to an endogenous “circadian” clock. Therefore, it is not surprising that many aspects of pharmacology and toxicology also oscillate according to the same 24-hour 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, but organized in hierarchical fashion. Interestingly, some aspects of physiology and behavior are controlled directly via a “master clock” in the suprachiasmatic nuclei of the hypothalamus, while 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 considering “chrono-interactions” among different clock-regulated systems.

Keywords

circadian rhythms; drug metabolism; chronotherapy; cancer; peripheral oscillators; systems biology

2 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 clocks are named “circadian” (Latin *circa diem* – 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. Historically, it was early recognized that rhythmic physiology resulted in rhythmic disease symptoms. Hippocrates already noticed ca. 400 BC that daytime sleepiness is indicative of disease, and nighttime sleeplessness can indicate pain and suffering (1). By medieval times, reports existed of daily variations in diseases such as bronchial asthma (2). For over thirty years, it has been known that drug absorption and distribution is subjected to diurnal

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The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

variation in rodents and humans. A twenty-four hour change in drug bioavailability has therefore been established for hundreds of drugs in rodents and humans. For example, acetaminophen (3) or theophylline (4) show different pharmacokinetics in the morning compared to evening. These changes are the results of several time-dependent modifications of physiological and molecular aspects that influence drug absorption and distribution.

Considering the wide scope of circadian (patho-) physiology, it is logical that the pharmacodynamics and pharmacokinetics (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, or 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.

3 Molecular fundamentals of circadian clocks

The basic unit of circadian timekeeping is the cell: even in very complex organisms, most cells contain autonomous circuitry for circadian oscillations. Generally speaking, this mechanism is comprised 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 insures this repression is short-lived, so that a new cycle can begin. In mammals, the principal activators within this system are the CLOCK and BMAL1 proteins and their homologs, which dimerize and bind to cis-acting E-box elements (with the simple consensus DNA sequence CAANTG) to activate 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 β represses *Bmal1* transcription in a parallel but interlocked loop. The ROR 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 is 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 to regulate chromatin structure and 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 parallel and independent circadian mechanism independent of transcription also exists in parallel to 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, though posttranslational clocks based on phosphorylation are well studied in bacteria (10).

3.1 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 “slave” oscillators of similar molecular mechanism in cells from other tissues. Multiple redundant signals have been described. These include direct signals like innervation by the autonomous nervous system and hormones like glucocorticoids, and 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 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 perhaps due to 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 shiftwork, the SCN will shift its phase much more quickly (within a day or two) than 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 (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). Secondly, increasing evidence suggests that chronic circadian dysphasing by itself has significant negative consequences for health; either for rodents subjected to laboratory conditions of chronic jetlag or shiftwork, or in 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 disease specific to a peripheral organ might affect clockwork within this tissue has not been studied at all, but could also be highly pertinent to pharmacology.

3.2 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, about ten percent of all transcripts in each tissue are regulated in circadian fashion (20; 21). In large part, this regulation occurs through the same *cis*-acting promoter elements that direct the rhythmic transcription of clock genes themselves, for example the E-boxes that serve as platforms for activation by CLOCK and BMAL1, and the RRE-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 PAR-bZIP family of factors: DBP, TEF, and HLF. The D-elements to which they bind control circadian expression of several families of genes, including liver metabolic regulators critical to circadian control of pharmacokinetics for a wide variety of drugs (22). Modeling studies suggest that simple combinations of these three elements – E-boxes, D-boxes, and RRE-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, while 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 through circadian stimulation of action/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 transcription of certain genes. For example, rhythmic glucocorticoid production results in rhythmic activation of glucocorticoid receptor, which binds to cognate GREs (glucocorticoid receptor elements) to activate or repress transcription (26). Likewise, circadian body temperature variation results in rhythmic occupancy of heat shock elements (HSEs) (27). The result is circadian transcription of specific genes due to cell-extrinsic influences, and 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 circadian fashion (31-33), and the number of proteins that are expressed in circadian fashion is greater than the number of transcripts where this question has been addressed (34). Major signaling molecules like 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 post-translational modifications vary in 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 to 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 to 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 (40), and CHK1/2 (41), and by control of circadian transcription of the *p16-Ink4a* locus through the clock-associated NONO protein. In the latter case, this circadian interaction has been demonstrated to be 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.

4 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.

4.1 Neurotransmitters and circadian behavior

Nearly all behaviors show diurnal patterns of activity. In most cases, these oscillations have been shown to manifest themselves independently of external environment or the sleep-wake cycle. For example, long-term memory shows a direct dependence upon the circadian oscillator: not only do rodents and humans learn better at certain times of day than another, but mice with a functional circadian system learn better than those without (44). Similarly, anxiety show a clear diurnal pattern that is modulated both by sleep-wake and by the circadian oscillator, and anxiety behaviors are elevated in mice lacking the *Period* clock genes (45). Even perception of multiple different types of pain varies in 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 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 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, daily increase in prefrontal cholinergic neurotransmission is observed even in the absence of the task (48). In general, the cholinergic system has been documented to play a critical role in this type of circadian “time-stamping” of behavior. It is sustained by circadian release of acetylcholine during the active phase of many mammals, accompanied by increase in choline acetyltransferase and decrease in acetylcholinesterase activity. Globally, availability of muscarinic acetylcholine receptors shows an inverse pattern to acetylcholine availability, with increased abundance during the quiescent phase of the 24-hour 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 oscillation of the circadian clock gene *Per2* in forebrain neurons, suggesting a role for dopamine 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 in particular REM sleep (53). Circadian release of GABA and glutamate – the principal inhibitory and excitatory neurotransmitters of the brain, respectively – in turn not only control behavior, but also hypothalamic hormone release to regulate many aspects of physiology (54).

4.2 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 function (56), as well as affecting 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 upon digestive function (59).

More generally, autonomous circadian clocks not only within the GI tract, but also in numerous other tissues, have considerable influences upon physiology and metabolism. For example, ablation of clocks in pancreatic islets results in diabetes because of defects in coupling of β -cell stimulus to insulin secretion (60), and local clockwork controls 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).

4.3 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 circadian fashion (64), and macrophages in turn stimulate immune responses in equally diurnal fashion with their own clocks (65). By contrast, far fewer reports exist of circadian B-cell activity, and indeed the oscillations documented in circadian gene expression in peripheral blood mononuclear cells is much lower in amplitude than that 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 actual expression of clotting factors like PAI-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 like inflammation, which plays a strong role in circadian variation in many diseases (69).

5 Circadian pharmacokinetics: oscillations in jejunal, hepatic, and renal systems

5.1 Rhythmic gastric and intestinal absorption

Drug transport and diffusion is highly dependent on gastric pH that regulates drug ionization and hydrophobicity. In most animal species including man (70), gastric pH present a strong circadian pattern influencing drug solubility. At the same time, gastric emptying after a meal (71) and gastrointestinal motility (72) present a higher speed during the day than at night, increasing in this way 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 in the beginning of the day also contributed to increased drug distribution in daytime in humans (74) (Fig. 2).

5.2 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 P450 cytochrome (CYP P450) family of enzymes. The phase II proteins conjugate drugs to a hydrophilic molecule in order to increase solubility. They help to make lipophilic compounds hydrophilic enough to facilitate their excretion into urine, bile, and faeces. These reactions are achieved mainly by sulfotransferases, UDP-glucuronotransferases, glutathione S-transferases, and N-acetyltransferases. Finally, phase III transporters – mainly 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 P450 oxydoreductase family. For example, Aminolevulinic Acid Synthase (ALAS1) is the rate-limiting enzyme in 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 oxydoreductase (POR) enzyme to the heme group (77).

Importantly for pharmacokinetics, expression of all of these proteins is carefully coordinated to favor efficient liver detoxification. 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 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 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, but rather suggest regulation by circadian clock-controlled transcription factors (80). Studies in genetically engineered mice revealed the pivotal role of the PARbZip transcription factors to achieve 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 of 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 or *Abcb1a*) (83). However, another important mode of activity also exists: PARbZip transcription factors also regulate 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 for the clock genes *Clock* and *Bmal1* themselves, since these mice also present low level of expression of PARbZip transcription factors (84). It is interesting to note that the time-dependent toxicity of pesticide in *Drosophila* involves similar mechanisms, through the regulation of the CAR ortholog DHR96 by the PARbZip ortholog *Pdp1* (85) and the rhythmic expression of phase II enzymes (86).

Despite the importance of PARbZip transcription factor in circadian pharmacokinetics, other clock-regulated genes are also involved in rhythmic drug detoxification. For example, ROR α - and ROR γ -knockout mice present a deficiency in expression of several phase I and phase II enzymes, probably also as a result of a perturbed expression of the CAR xenobiotic receptor (87). In addition, mRNA coding for the other xenobiotic receptors *Pxr* (88) and *Ahr*, and its heterodimerization partner *Arnt* (89), also present a rhythmic pattern of expression. In the latter case, AhR's main target *Cyp1a1* is not only rhythmically expressed (89), but also induced in a time-dependent manner by the AhR ligand 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) (90). This time-dependent induction of *Cyp1a1* requires a functional circadian clock (91). Finally, acetaminophen time-dependent toxicity seems to be a result of rhythmic expression of the CYP2E1 (92), due itself to the rhythmic inhibition of the HNF1 nuclear receptor by CRY1 on the *Cyp2e1* promoter (93). Whereas the relative importance of all these systems on global rhythmic drug detoxification in mouse liver is not yet clearly demonstrated, there is no doubt that the circadian clock is a major actor in this arena (Fig. 2).

5.3 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 subject to a second round of hepatic metabolism or fecal excretion. The hepatobiliary transport system is required not only for bile formation, but also elimination of various endo- and xenobiotics including cholesterol, phospholipids, and drugs (94). Depending on the nature of the molecule, a broad range of liver-specific export systems are involved. Bile is formed by excretion of bile salts (BS) and non-bile salt organic anions via ABC transporters. Monovalent BS are excreted via the bile salt export pump (BSEP or ABCB11), while 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 (PC), 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.

Excretion of bile acids, lipids, and xenobiotics into the bile follows a stringent circadian rhythm, at least in rodents (95), and clock involvement has been documented at multiple different steps. First, bile acid synthesis follows a stringent diurnal rhythm in both rodent (96) and human (97). Conversion of cholesterol into bile acids involved the rate-limiting cholesterol-7 α -hydroxylase (CYP7A1), whose rhythmic expression is directly regulated mainly through REV-ERB α (98-100). 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 is not clearly described for the moment (20). As a consequence, it has been observed that the biliary excretion of drugs, for example ampicillin (101) or flomoxef, presents a diurnal pattern in rats (102) and patients under percutaneous transhepatic biliary drainage (103) (Fig. 2).

5.4 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 the 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 present a circadian behaviour in different mammalian models.

Around 20% of the RBF is converted into the 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. Finally, filtered and secreted drugs can be passively or actively reabsorbed out of the urine into the blood. Because, the RBF is a key determinant of glomerular filtration and secretion, it is intimately associated with the elimination of most ionized drugs through urine. The RBF has been demonstrated to follow a circadian oscillation with a peak during the active phase (104). Although this rhythm is probably partially entrained by circadian arterial blood pressure and the cardiac output, the rhythmic RBF could also be generated by an intrinsic renal mechanism. For example, *Cry1/Cry2* knockout (105) exhibit disrupted activity of the reninangiotensin-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 condition of inverted blood pressure (104). Rhythmic GFR is also maintained in transplanted human kidneys, indicating that sympathetic innervation is not required for this rhythm (106). 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 (107). It has been shown that several of these transporters present a robust diurnal expression in the more distal nephron segments, namely in the distal nephron and the collecting duct (108). Moreover, expression of MRP4 (ABCC4) and 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. Expression of *Nhe3* mRNA and protein in the kidney exhibit a robust circadian rhythm in rodent, with the maximal expression in the middle of the active phase (109). 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 (Fig. 2).

6 Chronobiological implications for drug treatment

To what extent has the knowledge presented above translated to effective pharmaceutical interventions? The most obvious examples for successful chronotherapy are ones with

obvious time-of-day-dependent symptoms. Treatment of bronchial asthma has been tuned to exhibit maximum plasma levels at the time of highest occurrence of dyspneas, and therefore alleviate symptoms most effectively (Fig. 3). Similarly, blood pressures shows a sharp peak in the early morning, importantly coinciding with the peak for cardiovascular events (110), and an extended trough during the night. Both healthy normotensive and patients suffering from essential hypertension exhibit this variation (111). The L-type calcium channel blocker Verapamil, for example, uses extended an release formulation to have therapeutically effective plasma levels in the early morning after bedtime oral administration (112). In addition, such delayed release drugs have been beneficial for hypertensive patients that do not show a nocturnal dip in blood pressure, so called “non-dippers” (113). Non-dipping is a risk factor for congestive heart failure even in clinically normotensive subjects (114; 115).

As mentioned above, not only PK/PD parameters are modulated by time-of-day, but also drug metabolism. For example, the over the counter acetaminophen (analgesic N-acetyl-para-aminophenol, APAP) is a leading cause of drug-induced liver failure in the United States (116). APAP is exclusively metabolized by the CYP P450 system of the liver (117), and toxicity is dependent on generation of N-acetyl-p-benzoquinone imine (NAPQI) by CYP2E1 (93). APAP toxicity is time-of-day dependent (3; 92), but liver specific ablation of the clock in mice blunts this rhythm (84).

6.1 Cancer

While 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 the one hand, chemotherapeutics should be dosed high enough to be toxic to the cancer, but on the other hand the dose should be low enough to prevent serious damage to healthy tissue or organs. That means pharmacokinetics and dynamics operate in a tight therapeutic range. Under these pre-conditions, the variations introduced by the circadian system on multiple levels can be crucial. This is further complicated by the possibility that not only the healthy tissue has a clock but also the tumor. In vivo, this has been shown measuring the incorporation of P³² in tumors of terminally ill breast cancer (118). These results are in line with newer in vitro data from various human and mouse cancer cell lines like the human U2 osteosarcoma (119). This is an important factor because most cancer drugs are toxic only to dividing cells or have a mechanism of action which 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, while alkylating agents cross-link DNA at any phase of the cell cycle (120). In case of an arrhythmic tumor, as is the case 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 and might slow down tumor progression additionally by this mode of action (121). 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 it has been shown that 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, 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 if the compound is administered during the first part of the active dark phase (122). This rhythm has been suggested to be dependent on CLOCK:BMAL1 binding in B-cells (122), changes in CYP P450 enzyme activity and even more importantly higher reduced glutathione levels at night might contribute, as has been described for other alkylating agents such as cisplatin. In contrast, 5-

fluorouracil's first and rate limited 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 thymidilate synthase (TS), which is important for DNA synthesis (123). DPD and TS expression 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 (LV) is an inhibitor of TS and often co-administered with 5-FU. This adds to the effectiveness of 5-FU and interestingly changes the DPD to TS ratio in the same direction as observed at the optimal time of day established in animal experiments.

Interestingly, there are further common traits between irinotecan and 5-FU. For both, added value of chronotherapeutic treatment regimes is gender-specific in experimental animal models. In the case of 5-FU this has even been observed in clinical populations (124). While chronomodulated delivery of 5-FU improves survival for male patients compared to conventional treatment, the opposite was true for female study participants. The authors speculate that since disruption of the rest/activity rhythm during chemotherapy has been shown to predict overall survival for metastatic colorectal cancer (125), the men could exhibit more robust circadian rhythms. However, further investigations are needed.

6.2 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 different times of day in order to assess if, for example, a certain type of receptor or protein is only expressed at a specific time of day. However, there are online resources that can be mined for information about the circadian expression of a given transcript or metabolite (126; 127). A special case presents in the quest for drugs against ageing related diseases. Similarly to human beings, rodent species typically used in these assays exhibit attenuated circadian rhythms. Thus, the PK/PD profile and target availability itself could change during the course of ageing.

Once the target is confirmed, and the lead optimization process started, the properties of the novel chemical entities are evaluated and selected. Typically, CYP P450 induction and inhibition in human and rodent primary hepatocytes is tested. This might introduce bias towards only one phase of the circadian cycle, since 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 not only used to predict absorption after oral application through the intestinal barrier but also to assess interactions with important transporters such as P-gp (128). Interestingly, like the intestinal barrier itself, the human tumor derived CACO-2 cells have a functioning clock (129) that has been shown to directly control 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 marketed drugs to be withdrawn are liver toxicity and cardiac safety. In fact, QT prolongation often associated with blockade of the K⁺-channel encoded by human ether-à-go-go related gene (hERG) and a surrogate marker for torsade des pointes (130) was the single most common cause for withdrawal of marketed drugs in recent years (ICH webpage) (131). Therefore, an extensive battery of tests from in vitro channel function to in vivo electrophysiology in the freely moving dog or monkey has been established (ICH7b, 2005) that is performed before a so-called "thorough QT/QTc" (ICH E14, 2005) study in Phase I of development. Although new models have been developed that adjust for circadian variability due to changes in heart rate over the day (132; 133), the possibility that drug dependent QT-interval prolongation is directly influenced by time of day in patients

has not yet been fully explored, but is not unsupported (134). Moreover, there is a clear rationale for how the circadian clock would influence cardiac repolarization, namely *krüppel-like factor 15* (135). 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, based on these considerations, drug developers would adopt a circadian testing policy, there is a further complication. In contrast to most pre-clinical animal models, there is a great deal of inter-individual variability in the circadian phenotype of people: period or phase and amplitude of clock-controlled rhythms described above varies greatly in human populations (136). These are no small differences, and the lay terms “larks” and “owls” for people with an early and late activity phase, 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 (137), and not only are the cortisol levels in depressed patients elevated but their diurnal variation is blunted (138). Twin studies suggest that genetic traits are partially determining the chronotype (139). In fact, there are multiple loci that have been determined to contribute to differences in chronotype and sleep (140). Moreover, there are quite consistent age-dependent changes of chronotype (141), and recent results suggest relatively stable changes dependent on previous light history (142).

Inter-individual differences in clock phase are sizable and therefore probably clinically relevant. Albeit significant methodological advances have been made to determine the properties of an individual's circadian clock in a simple test, this goal 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 in this direction (143), and hair follicles samples have also been used (144). Alternatively, clock-controlled neuroendocrine signals such as the dim-light melatonin onset (DLMO) have been used to estimate clock phase (145). Due to large inter-individual variation in the levels of these hormones between individuals multiple sampling is necessary to establish meaningful results, which is also dependent on ambient light levels. To overcome these limitations, transcriptome and metabolome datasets have been used to establish timetable methods for internal time in mice and men (146-148). All of these methods, however, rely on at least two sampling times that are optimally 12 hours apart to compensate for inter-individual difference in the absolute levels of gene or metabolite expression, and “internal time” can be approximated with 2 hours' precision. Thus, a feasible and accurate circadian test remains to be established.

Given aforementioned examples of arrhythmicity in disease and inter-individual differences in clock phase, the circadian clock itself might be an interesting target for drug development. In fact, multiple pharmacological agents have been reported to phase-shift behavioral and biochemical rhythms in experimental animal models and people, and for example melatonin, melatonin agonists, or a combined melatonin agonist/5-HT_{2c} antagonist have already received market approval, although not necessarily as phase-shifting drugs. In addition to the melatonin receptors, several independent molecular targets have been identified to alter clock function. Amongst the first that was identified was *casein kinase 1*, and even subform-specific tool compounds have been described (149). As a target, however, kinase inhibitors are not without problem. *Casein kinase 1* is, for example, involved in *Wnt* signaling and linked to cell proliferation and survival (150).

Furthermore, a number of compounds that target core molecular clock components or are believed to be important links between the clock and HPA axis have been identified and tested, including a Neuropeptide Y Y5 receptor (151), a tachykinin antagonist (152), an inverse agonist of ROR α (153), and a corticotrophin releasing factor (CRF) antagonist (154). 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 α (155-157). Whether these in vitro data from U2OS cells will be able to translate in the in vivo situation will be very interesting.

More general, it remains to be proven if the clock does indeed present a “druggable” target, or if there are unsuspected mechanistic problems in altering circadian rhythms. Should such compounds exhibit safety profiles as favorable as melatonin, for example, they could prove useful in a wide spectrum of possible indications, ranging from sleep/wake problems in shiftworkers to amplitude-related problems in ageing related diseases. On the one hand such drugs could help to “boost” circadian rhythmicity beyond what can be achieved through behavioral measures, and on the other hand re-adjust specific rhythmic components to a favorable phase. This would be especially useful given the tendency of modern society towards a 24/7 lifestyle.

In this respect, more and more evidence has accumulated showing the sometimes disastrous effects of such clock disruption on health (15; 16; 18; 19; 158). 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 feedback 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, either the use of hypnotics and scheduled meals can normalize circadian gene expression rhythms and improve disease symptoms (159). Similarly, melatonin and bright light treatment have been shown to have a positive effect on institutionalized Alzheimer patients (160).

7 Conclusions

The recent findings that we have highlighted all 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 expression and functional properties of drug metabolizing enzymes and drug transporters are highly species-specific (161), extrapolation of these results to humans is not a foregone conclusion. In order to translate research data into clinical application, significant progress in the characterization of circadian variations in protein expression and activity in human 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 seem to not have translated into clinics or regulatory agencies on a broad scale. Publicly available clinical trials databases such as clinicaltrials.gov list a historic total of 205 hits for the search term “circadian”. Twelve of these are cancer-related but none try to establish chronotherapeutic treatment regimes. The search term “chronotherapy” results in 14 hits. In contrast, the search term “cancer” produces 38331 results. Similar results were obtained from the EU clinical trials register. Given the fact that about 20% of the transcriptome, proteome and metabolome are under clock control (34; 162; 163), this seems disproportionate. In the case of regulatory authorities, none of the chronobiological effects upon PK/PD outlined here are mentioned in 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 withdrawal of marketed drugs.

Finally, the large proportion of physiology regulated by the circadian clock suggests that the clock itself might present a possible pharmaceutical target to increase efficacy and reduce side effects. In order for such treatments to be effective, a 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.

Acknowledgments

Research support for the laboratory of Steven A. Brown comes from the Swiss National Science Foundation, the Velux Foundation, the Swiss Cancer League and the Clinical Research Priority Program Sleep and Health. The laboratory of Frédéric Gachon is supported by the European Research Council and the Leenaards foundation.

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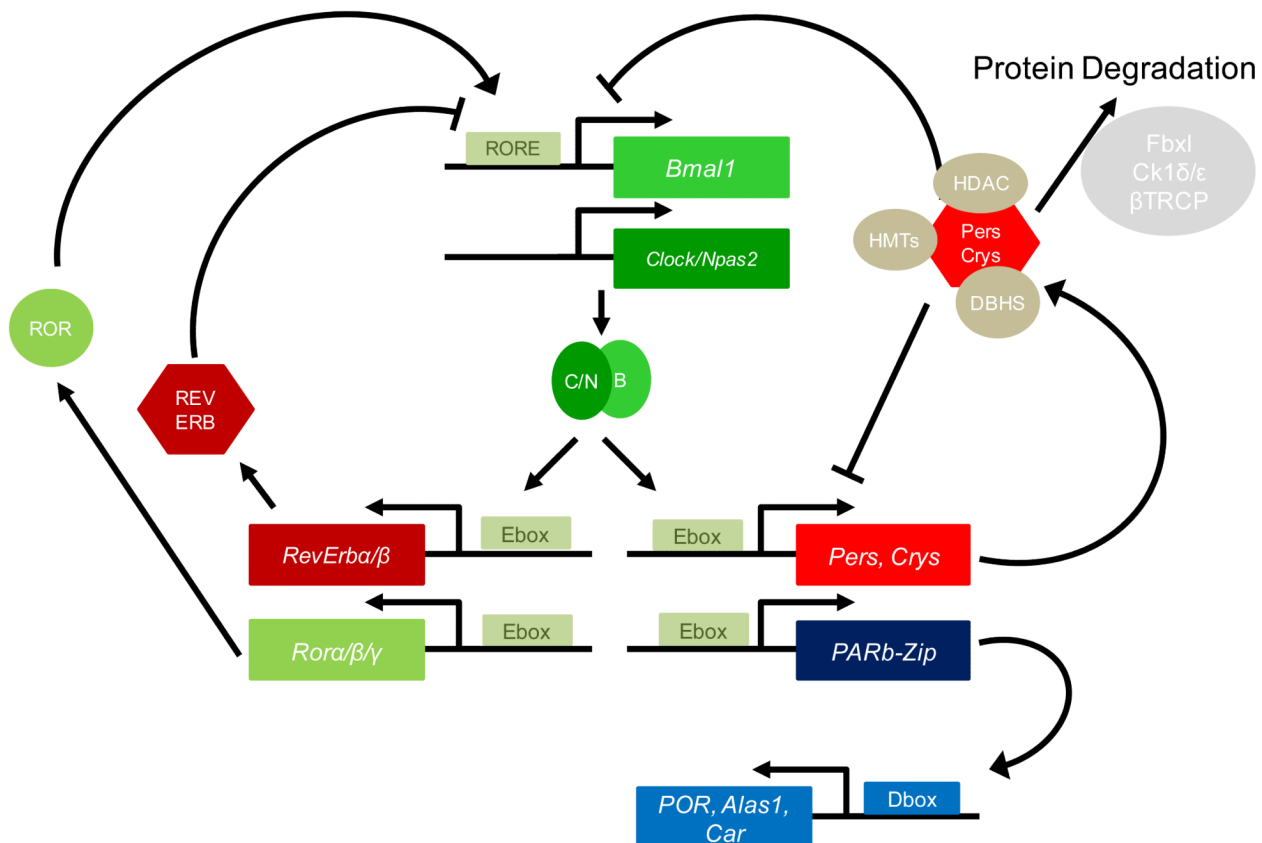


Figure 1. The canonical mammalian circadian oscillator and output relevant for xenobiotic metabolism

Two interlocked feedback loops composed of activators (green) and repressors (red) that drive gene 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), DBHS family RNA-binding proteins (beige), and kinases and proteasome machinery (grey). Important output genes, involved in transcriptional control of the detoxification metabolism (blue). See text for details.

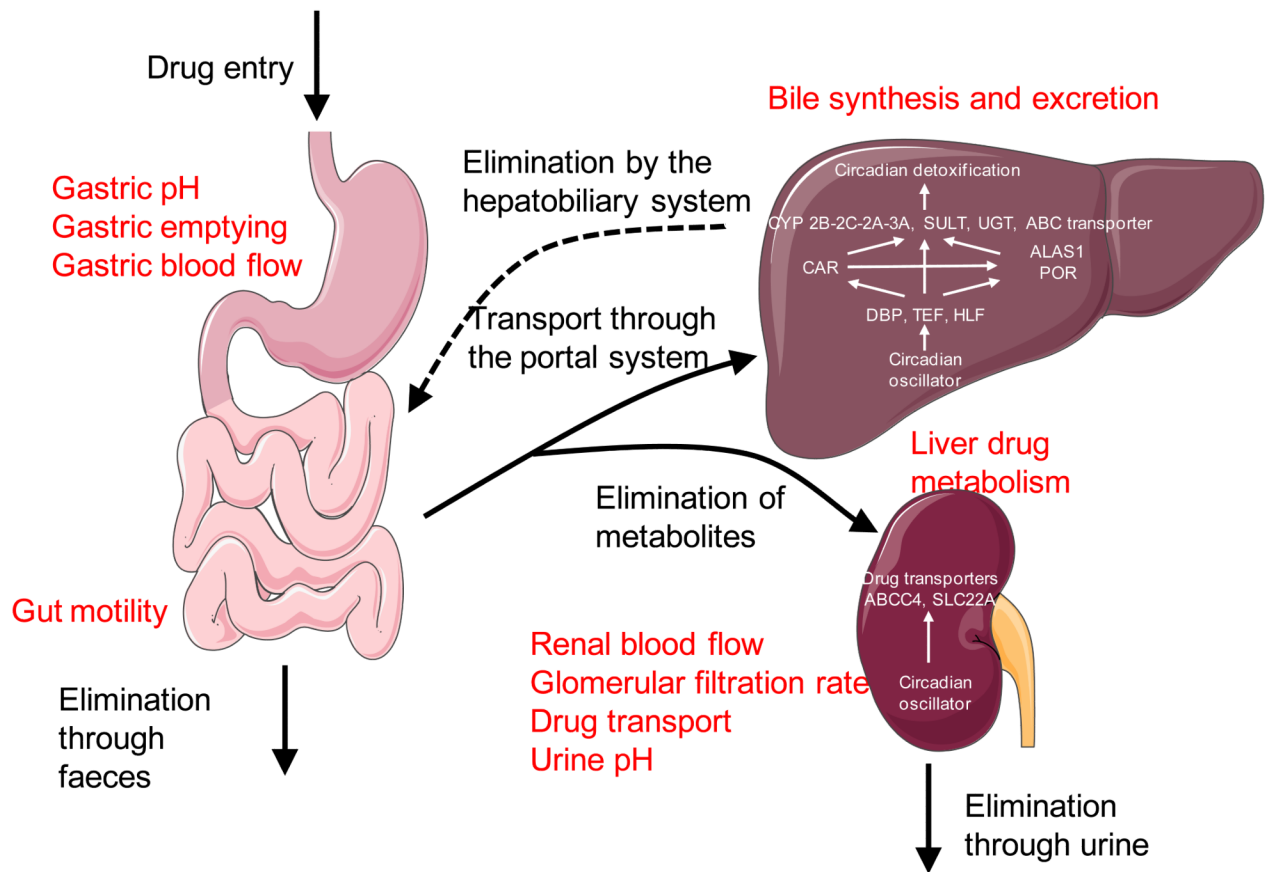


Figure 2. Modulation of drug pharmacokinetic by the circadian clock

All rhythmic parameters influencing drug transport and metabolism are highlighted in red. Characteristic functions of the circadian clock on these process are indicated in the respective organs.

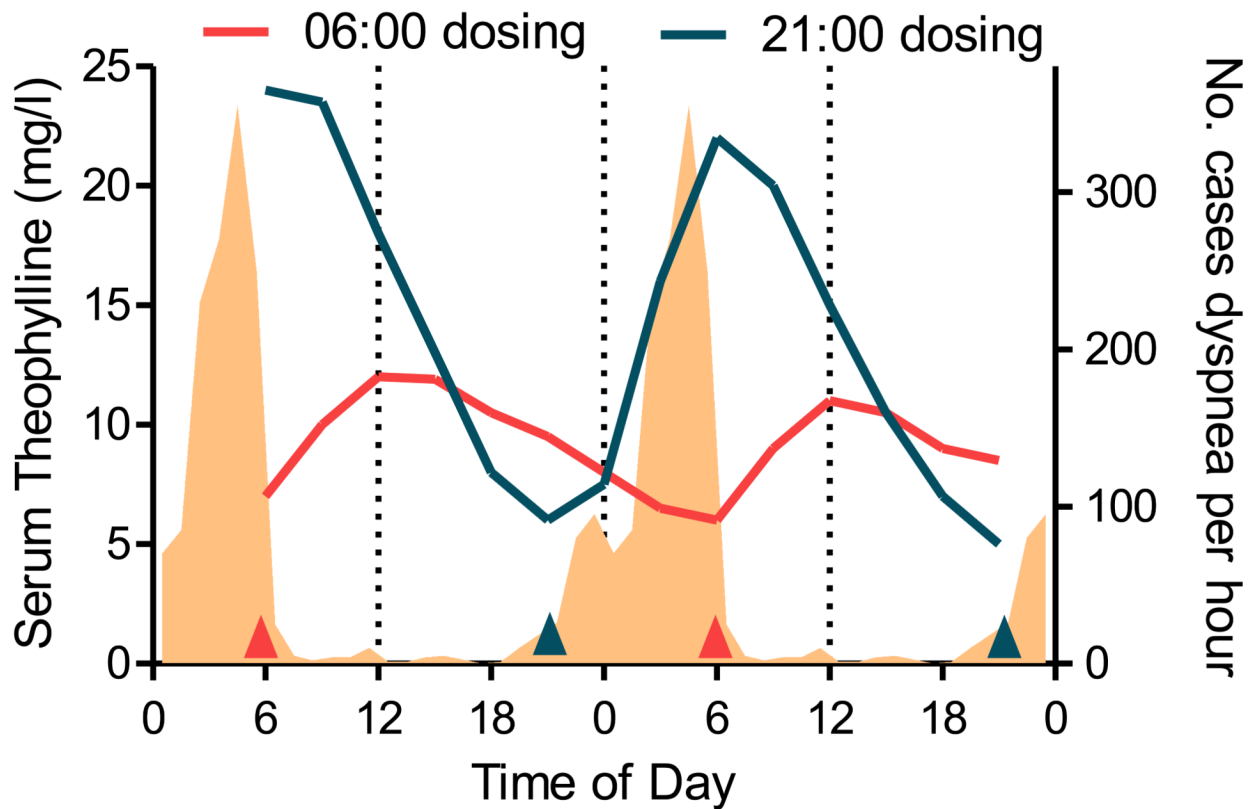


Figure 3. Time of day dependent variation in pharmacokinetics.

Average 3-hourly steady state serum concentrations of theophylline from eight asthmatic children dosed with Theo24© before breakfast (06:00, red) or at bedtime (21:00, blue) as indicated by arrows. Shaded area represents the occurrence of dyspnea. (Adapted from Smolensky et al 1987 and Dethlefsen & Repges (1985) c.f. Smolensky et al. (1987))