Circadian Rhythms: Mechanisms and Therapeutic Implications

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Abstract

The mammalian circadian system is organized in a hierarchical manner in that a central pacemaker in the suprachiasmatic nucleus (SCN) of the brain's hypothalamus synchronizes cellular circadian oscillators in most peripheral body cells. Fasting-feeding cycles accompanying rest-activity rhythms are the major timing cues in the synchronization of many, if not most, peripheral clocks, suggesting that the temporal coordination of metabolism and proliferation is a major task of the mammalian timing system. The inactivation of noxious food components by hepatic, intestinal, and renal detoxification systems is among the metabolic processes regulated in a circadian manner, with the understanding of the involved clock output pathways emerging. The rhythmic control of xenobiotic detoxification provides the molecular basis for the dosing time-dependence of drug toxicities and efficacy. This knowledge can in turn be used in improving or designing chronotherapeutics for the patients who suffer from many of the major human diseases.

INTRODUCTION

The availability of new drugs and the better use of existing ones represent essential assets for improving human health through prolonged disease control or cure without enduring the adverse events that compromise quality of life or survival (1). However, the recent recognition of late severe drug toxicities emphasizes the need for new tools and methods that improve the safety of treatment (2). In addition, the more than tenfold inter- and intrapatient variability in the relationships between drug dose and pharmacokinetics, toxicities, or activities is common and calls for complementary approaches in pharmacology and therapeutics (3–5). Improving and individualizing drug doses and schedules is even more important for the treatment of chronic diseases, which represent the major cause of human morbidity and mortality worldwide (6). Although pharmacogenomics aims at predicting interpatient differences in drug metabolism, toxicity, or efficacy, rapid advances in our understanding of molecular clocks and their signaling pathways are now providing both new targets and new time cues for therapeutic interventions.

Somewhat arbitrarily, biological clocks are commonly subdivided into ultradian ($\tau < 20$ h, $\tau =$ period length), circadian ($\tau = 20$ to 30 h, circadian is derived from the Latin words circa diem, about a day), and infradian oscillators ($\tau > 30$ h) (7). In this review, we limit the discussion to the circadian system, as the possible relevance of ultradian and infradian clocks for drug metabolism has not yet been studied in detail. Virtually all light-sensitive organisms from cyanobacteria to humans possess circadian clocks, suggesting that such devices offer a selective advantage. Many behavioral and physiological facets of life profit from a circadian organization. Behavioral traits include the anticipation of daily food availability and predator avoidance. The temporal sequestration of chemically incompatible reactions—such as photosynthesis and nitrogen fixation in cyanobacteria or the synthesis and degradation of glycogen as energy stores in mammalian liver and muscle tissue—may be part of the physiological purpose of a circadian organization. Furthermore, and particularly relevant for the subject covered in this review, the physiological adaptation to daily food uptake, metabolism, and detoxification is regulated in an anticipatory fashion by the mammalian circadian timing system (8). However, the utility of circadian clocks under laboratory conditions has been shown in a compelling fashion only for cyanobacteria and green plants. Specifically, cyanobacteria with endogenous oscillators that resonate with environmental light-dark cycles outgrow cyanobacteria with nonresonating clocks in competitive culture conditions (9, 10), and *Arabidopsis thaliana* plants with resonating oscillators perform photosynthesis more efficiently and are more resilient to environmental insults than plants with nonresonating circadian time keepers (11).

Rosbash & Gehring have recently hypothesized that the evolution of circadian clocks in the early metazoan period was for the avoidance of genotoxic UV light (12). Primitive organisms belonging to the marine zooplankton may have used blue light receptors (e.g., photolyases and cryptochromes) to avoid UV-rich sunlight by moving daily to deeper sea levels. These blue light receptors later evolved to become coupled to a circadian oscillator that could anticipate this up-and-down migratory

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behavior. Unlike most biochemical reactions, the ones generating daily rhythms are temperature-compensated. That is, within the physiological temperature range, the temperature coefficient Q_{10} for period length lies within narrow boundaries (0.8 to 1.2) (13). Obviously, temperature compensation of circadian timing is essential in all organisms incapable of maintaining a uniform body temperature. The mechanisms assuring temperature compensation have not been dissected in molecular detail, but at least in cyanobacteria, this should now become possible. In fact, a temperature-compensated circadian oscillator governing daily protein KaiC phosphorylation cycles during almost a week can now be reconstituted in the test tube with just three purified proteins (KaiA, KaiB, KaiC) and ATP (14).

The major objective of this review is to describe the role of the mammalian circadian timing system in coordinating xenobiotic detoxification and, as a consequence, drug metabolism. By using two examples for the chronotherapeutic treatment of chronic disease types, we illustrate how this knowledge can be used for the design of better drug delivery regimens in the clinic.

THE MAMMALIAN CIRCADIAN TIMING SYSTEM

Circadian Physiology

The main task of the circadian timing system can be viewed as the optimization of metabolism and energy utilization for sustaining life processes in the organism. In this context, it is perhaps not surprising that most mammalian physiology is influenced at least to some extent by the circadian pacemaker (15). For example, rest and activity, heart rate, blood pressure, liver and renal plasma flows, bile and urine production, intestinal peristalsis, secretion of digestive enzymes into the gastrointestinal tract, major endocrine functions, and metabolism are all subject to daily oscillations. The mammalian circadian timing system has a hierarchical structure, in that a master pacemaker residing in the suprachiasmatic nucleus (SCN) of the brain's hypothalamus orchestrates countless clocks in most peripheral cell types (16, 17). This organization implies that circadian manifestations in behavior and physiology can result from cyclic neuronal and humoral signals emanating directly or indirectly from the SCN, or from rhythms in gene expression or enzymatic activities governed by local circadian clocks in peripheral cells. The molecular makeup of circadian oscillators operative in SCN neurons and peripheral cell types, such as hepatocytes, fibroblasts, or muscle cells, is very similar (18–20), and the only major differences between them lie in their hierarchical position and in the ways they are synchronized. While the phase of SCN pacemakers is entrained by daily light-dark cycles perceived by the retina (21), the phase of peripheral oscillators is adjusted by chemical zeitgebers, i.e., periodic signals that provide time cues to living organisms, tissues or cells (15), in particular those generated by feeding-fasting rhythms. The synchronization of circadian oscillators in many tissues by feeding cycles lends support to the notion that the timing of metabolism is a major function of the circadian timing system. In this context, the daytime-dependent inactivation of noxious food components is particularly

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relevant. The underlying xenobiotic detoxification system plays an important role in the timing of drug metabolism, manifesting itself in circadian pharmacokinetics and pharmacodynamics, hence producing circadian changes in drug effectiveness and toxicity. Mouse or rat models have shown that the same dose of a drug can be lethal when administered at certain times of day or night, but has little adverse effect when given at other times (22–24). In humans, modifications in drug timing often result in predictable changes in drug pharmacology that may translate into clinically relevant differences in treatment effects (24). However, the extent of individual benefit from circadian-timed therapy can differ as a function of environmental, genetic, and epigenetic factors.

Model for Mammalian Molecular Oscillator

During the past decade, considerable progress has been made in the molecular dissection of mammalian circadian oscillators; **Figure 1** displays a simplified scheme for the circadian oscillator that currently serves as a working model in several laboratories (16). According to this scheme, circadian oscillations are generated by transcriptional and posttranscriptional feedback loops involving a positive limb and a negative limb. The positive limb consists of the three transcription factors BMAL1, CLOCK, and NPAS2, a closely related paralog of CLOCK. BMAL1-CLOCK or BMAL1-NPAS2 heterodimers activate members of negative limb, such as cryptochrome (*Cry*) and period (*Per*) genes by binding to E-box elements within enhancer and promoter sequences of these genes. As a consequence, CRY and PER accumulate to levels high enough to form heterotypic complexes that attenuate the transactivation potential of BMAL-CLOCK/NPAS2 heterodimers and thereby autorepress their own genes. This leads to a decrease of CRY and PER levels below the concentration required for autorepression, and a new cycle of *Cry* and *Per* transcription can ensue. The same positive (BMAL1, CLOCK, NPAS2) and negative transcriptional regulators (CRY and PER proteins) also drive the circadian expression of the orphan receptor REV-ERBα, a strong repressor of *Bmal1* transcription and a moderate repressor of *Clock* transcription (25). REV-ERBα and, perhaps, its paralog REV-ERBβ couples antiphasic transcription cycles of negative and positive limb members. Although this coupling is dispensable for rhythm generation, it contributes to the robustness and phase shifting properties of the clockwork circuitry. As indicated in **Figure 1**, additional components are required for proper clock function, such as the WD40 protein WDR5, which serves as a binding platform for histone methyl transferases; the RNA- and DNA-binding protein NONO, which affects circadian rhythm generation in an unknown fashion; and the protein kinases CK1ε/δ and CK2, which modulate the stability and activity of both positive and negative limb members (26–31). To date, *Bmal1* is the only clock gene whose inactivation immediately results in behavioral arrhythmicity (32). All other known clock components are represented in the genome by at least two isoforms (e.g., *Per1* and *Per2*, *Cry1* and *Cry2*, *Rev-erbα* and *Rev-erbβ*) (25, 33–35). The CLOCKΔ19 mutation discovered by Takahashi and coworkers is generated through exon 19 skipping (36). Although this dominant negative transcription factor strongly compromises circadian clock function, mice with a *Clock* null allele display

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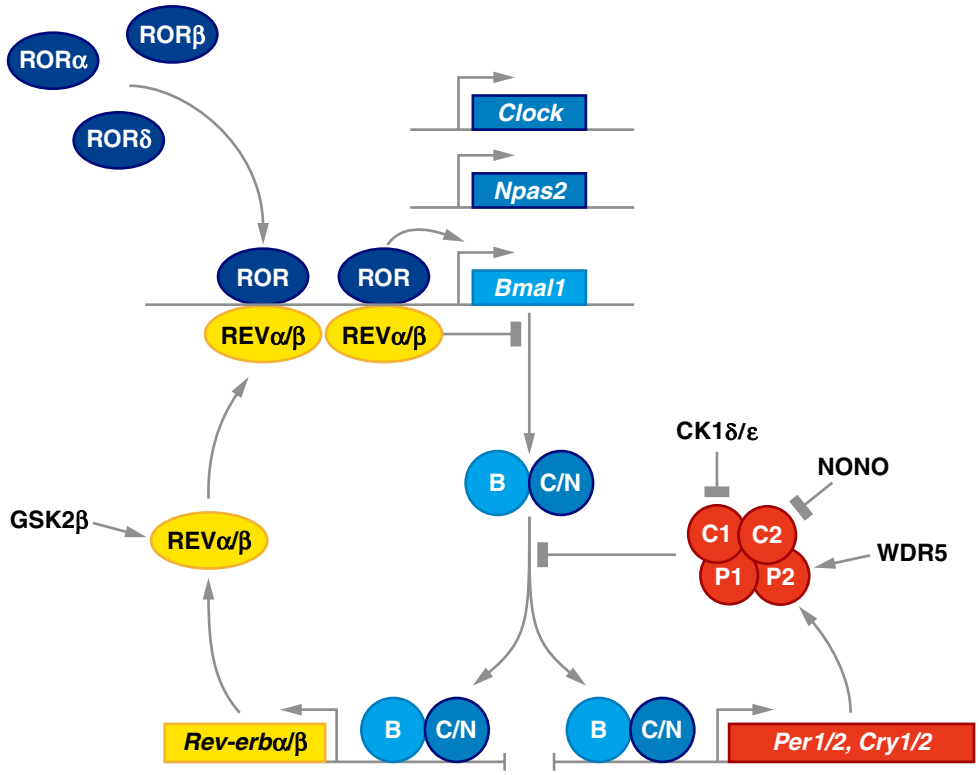

Figure 1

Simplified hypothetical model of the mammalian circadian oscillator. The molecular oscillator of mammalian cells is thought to be based on molecular feedback loops within a positive limb (CLOCK, BMAL1) and a negative oscillator limb (PER and CRY proteins) that are interconnected via the nuclear orphan receptor REV-ERBα. It is not yet certain whether the interactions shown are indeed the rhythm-generating principle or whether they are part of a clock output pathway translating oscillations driven by a yet unknown oscillator (e.g., based on feedback regulation of enzyme activities) into overt rhythms of gene expression (for explanations, see text).

relatively mild circadian phenotypes (37). Hence, other proteins, such as NPAS2 (38), probably assume the role of CLOCK in these mutant animals. CLOCK has recently been demonstrated to have histone acetyl transferase activity, a common signature for transcriptional coactivator protein complexes (39). It is therefore also conceivable that structurally related coactivator proteins, such as the basic helix-loop-helix SRC proteins SRC1, TIF2/GRIP1, and ACTR (40), may substitute for CLOCK in *Clock* knockout mice.

It should be emphasized, however, that the model depicted in **Figure 1** suffers from several intrinsic inconsistencies that will have to be resolved in the future (8, 41). For example, the phase relationship between *Per* and *Cry* mRNA and protein accumulation does not correspond to that expected for a direct autoregulatory feedback loop.

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Moreover, in other systems, there is increasing evidence that regulatory components governing posttranslational steps, rather than transcription factors, may be the central cogwheels of the circadian clockwork (for review, see Reference 42). Therefore, we consider it possible that the gene expression circuitry depicted in **Figure 1** may control output functions of the circadian timing system rather than the generation of the cycles per se.

The Hypothalamic Master Pacemaker and Peripheral Clocks

The analysis of cyclic gene expression in tissue explants and cells cultured in vitro has demonstrated that circadian oscillators function in a self-sustained and cell-autonomous fashion (20, 43, 44). Although these cellular oscillators are coupled in the suprachiasmatic nucleus, they do not seem to be so in cultured fibroblasts (43) or in peripheral organs of SCN lesioned animals (45). Hence, it is likely that the SCN synchronizes individual cells rather than entire tissues or organs in the intact animal.

The role of the SCN as a central circadian pacemaker was established almost two decades ago through elegant lesion and transplantation experiments in laboratory rodents (46). Thus, bilateral lesion of the SCN in hamsters immediately resulted in behavioral arrhythmicity under constant conditions (DD for dark-dark). More interestingly, implantation of fetal SCN tissue restored circadian locomotor activity within a few days to weeks. When genetically different hosts and donors that free-run with a different period length in DD were used in these lesion-transplantation experiments, the donor tissue dictated period length. Subsequent studies revealed that even SCN tissue encapsulated into porous plastic was capable of restoring circadian behavior in SCN lesioned hamsters (47). Therefore, diffusible signals emanating from the SCN must play an essential role in governing daily rest-activity cycles. Three SCN-derived signaling polypeptides, transforming growth factor alpha (TGFα) (48), prokineticin-2 (PK-2) (49), and cardiotrophin-like cytokine (CLC) (50), fulfill the criteria expected for such signaling cues because they suppress locomotor activity when infused into the brain's third ventricle. Moreover, CLC antibodies generate additional locomotor activity when infused into the brain's third ventricle. SCN tissue grafts from wild-type mice can restore circadian locomotor activity when implanted into genetically arrhythmic *mCry1-mCry2* double knockout mice. Therefore, peripheral oscillators do not appear to be required for the generation of circadian locomotor activity (51). However, in a natural light-dark environment, peripheral oscillators may also be coordinated through SCN-independent pathways because similar rhythms were found for bone marrow DNA synthesis and for liver dehydropyrimidine dehydrogenase mRNA in mice with intact or ablated SCN (52). As shown below, however, the local oscillators also play crucial roles in the circadian metabolism of xenobiotic substances, and hence in chronopharmacology and chronotherapy.

In rat, each of the two SCN is composed of approximately 10,000 neurons and an unknown number of astroglia cells. The SCN is generally separated into a two anatomically and functionally different regions—the core and the shell—and each of these subregions contains a number of different specialized neuron types with regard to neuroendocrine functions (53). However, the SCN also regulates circadian

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physiology through synaptic connections to various brain nuclei and, through the autonomic nervous system, to peripheral organs (54). For example, light perceived by the retina can signal the release of glucocorticoid hormones in the adrenal cortex via the SCN and the splanchic nerve without a functional hypothalamus-pituitary gland-adrenal axis (55). Each SCN neuron contains its own cell-autonomous circadian oscillator. Surprisingly, however, the period lengths measured for individual cells display a very wide range, from less than 20 h to more than 30 h (56). In the intact SCN, the neurons must therefore be coupled by synaptic and paracrine signaling to keep them synchronized. The VIP/PACAP receptor VPAC2 (57) and the cell adhesion molecule NCAM-180 (58) appear to be implicated in paracrine signaling pathways required for synchronizing neurons within the SCN.

As the circadian oscillator generates rhythms whose period length deviates by a few minutes from the 24 h day, they must be tuned daily to geophysical time by an input pathway. Daily light-dark cycles are clearly the major zeitgeber for the SCN master clock. Light signals are detected by classical rod and cone photoreceptor cells as well as melanopsin-containing retinal ganglion cells, and they are transmitted as electrical signals to SCN neurons via the retino-hypothalamic tract (59). This synaptic transmission involves the neurotransmitters glutamate and PACAP and provokes the influx of Ca^{2+} and the activation of various protein kinases, such as CaMKII, MAPK, and PKGII. The activated protein kinases then phosphorylate CREB and possibly other transcription factors that stimulate the transcription of immediate early genes. PER1 and PER2 play a pivotal role in the light-entrained synchronization of SCN oscillators because they serve as both core clock components and immediate early proteins (60, 61). Whereas the light-induced stimulation of *Per2* expression during the early night elicits phase delays, light-induced enhancement of *Per1* expression during the late night facilitates phase advances (60). Phase shift response curves (PRC) obtained by delivering light pulses across the circadian day have been established for many species, including humans (62). For the latter, maximal phase delays and phase advances amount to approximately 3 h and 2 h, respectively. This limited phase shifting capacity of the SCN pacemaker explains why we suffer from jet lag during several consecutive days after east- and west-bound transatlantic flights.

Peripheral Clocks

The presence of clocks in peripheral mammalian cells has been demonstrated by measuring circadian gene expression in cultured fibroblasts or tissue explants (18, 20, 43, 44). In vitro studies on cell populations or organ slice cultures exposed to a single serum shock (treatment with 50% horse serum for two hours) first suggested that peripheral oscillators dampen with time. These oscillators were thus believed to require daily systemic signals from the SCN to maintain both amplitude and phase in intact animals (18). However, recent observations on individual cultured fibroblasts changed this view (43, 44). In fact, circadian oscillators in cultured fibroblasts are self-sustained and cell autonomous, just like the ones operative in SCN neurons. Daily oscillations in clock gene expression even continue during mitosis, during which the circadian phase is passed on to the two newborn daughter cells (43). Because the

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period length varies between cells and fluctuates during the lifetime of an individual cell, the amplitude of rhythmic gene expression appears to decrease progressively when measured in cell populations synchronized with a single pulse of a strong phase-shifting agent (see below).

The molecular nature of the zeitgeber signals responsible for keeping peripheral oscillators tuned in intact organisms is still unknown. Attempts to identify such signals in serum failed for an unexpected reason. In fact, virtually every signaling pathway examined is capable of synchronizing circadian fibroblast clocks (for review, see Reference 8), and it is thus difficult to purify chemical signaling substances from complex body fluids such as serum. We thus feel that genetic approaches will be required to identify critical components implicated in the synchronization mechanisms operative in vivo. For example, genes whose transcription is still rhythmic in clock-deficient organs of animals with an intact master SCN clock are likely to be system-driven. If these genes are coupled to the oscillator, they may convey the phase dictated by the SCN to local circadian clocks and hence serve as mediators between central and peripheral timekeepers. A mouse strain with a conditionally active liver oscillator has recently been established (B. Kornmann & U. Schibler, unpublished results), and at least for hepatocyte oscillators, such experiments can now be initiated.

Endeavors to delineate molecular phase-entrainment pathways by genetic approaches will be aided by the observation that feeding-fasting cycles are dominant zeitgebers for many peripheral oscillators (63, 64). For example, if nocturnal rodents are fed exclusively during the day for a week or longer, the phase of circadian gene expression becomes completely inverted in many peripheral organs. As feeding time has little impact on the phase of the SCN pacemaker, the peripheral timekeepers become uncoupled from the master clock upon daytime feeding. However, as soon as the restricted feeding regimen is terminated and food is given back ad libitum, the phase of peripheral oscillators is switched back to normal within two days. This demonstrates that clocks in peripheral tissues are capable of large phase shifts under certain conditions, a property that can also be demonstrated with cultured fibroblasts (43). At least in part, glucocorticoid signaling accounts for the different kinetics associated with inverting the phase of peripheral clocks by restricted feeding and shifting it back to normal after the conflicting feeding regimen has been terminated. Thus, in the absence of corticosterone or glucocorticoid receptor, daytime feeding inverts the phase in peripheral organs in only two to three days (65). Body temperature rhythms may also contribute to the phase entrainment of peripheral clocks, albeit in a less dominant fashion than feeding-fasting cycles (66).

Transcriptome profiling in several tissues have indicated that between 2% and 10% of genes are transcribed in a circadian manner (67–70). In liver, mRNAs encoding regulators and enzymes relevant for the metabolism of carbohydrates, proteins, lipids, and xenobiotic substances display daily accumulation cycles (68, 71). Whether circadian mRNA accumulation results in circadian protein accumulation depends on protein half-life. Even a large periodic burst in mRNA levels does not necessarily result in significant protein oscillation if the protein in question is metabolically stable. Conversely, translational and posttranslational mechanisms can elicit circadian activity rhythms of proteins translated from mRNAs whose levels do not vary

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during the day (72). For example, the core clock protein CRY2 accumulates in a strongly circadian manner in spite of nearly invariable levels of *Cry2* mRNA (25). Conceivably, the metabolic stability of CRY2 is influenced by its interaction with PER proteins, which are subject to strong daily oscillations. Even proteins whose abundance is invariable may act in a circadian fashion because their activity may be influenced by daytime-dependent posttranslational modifications, interactions with ligands and cofactors, or protein partners. Thus, the glucocorticoid receptor activates genes rhythmically because its ligand fluctuates during the day (73), and some constitutively expressed cytochrome P450 (CYP) enzymes may have a rhythmic activity because P450 oxidoreductase—which provides the electrons required for monoogenases reactions—accumulated in a rhythmic fashion (71).

CHRONOPHARMACOLOGY

Dosing Time Dependencies in Drug Effects

The widespread awareness of the importance of the time of day on pharmacology can readily be illustrated by a literature search. As of May 2006, entering "pharmacology" and "circadian" into PubMed resulted in more than 14,000 references, including approximately 1200 review articles. The influence of the time of day on drug efficacy and toxicity is perhaps not surprising considering that most mammalian physiology is affected by the circadian clock. Currently, most approaches consider the relevance of dosing time for drug effects to recommend a standard best time for drug administration in populations with well-synchronized circadian physiology. Recently acquired knowledge on the circadian timing system and the availability of new experimental and computational models and technologies now allow for the identification of the key clock and clock-controlled components that influence the dynamics of drug effects. In turn, the variability factors that impinge on these biological timers and their signaling pathways can now be studied much more precisely.

The lethal toxicity of a fixed dose of a drug has long been known to vary as a function of dosing time in laboratory rodents irrespective of drug class (74). For instance, the lethal toxicity of mice exposed to halothane fluctuates between 5% and 76%, depending on when the animals were exposed to the drug (75). Two- to tenfold changes further characterize the tolerability of mice or rats as a function of dosing time for more than 35 anticancer drugs, including antimetabolites 5-fluorouracil (5-FU) (76) and L-alanosine (77), DNA intercalators doxorubicin (78) and theprubicin (79), alkylators cyclophosphamide (80) and oxaliplatin (81), mitotic inhibitors vinorelbine (82) and docetaxel (78), interferon-α (83), COX-2 inhibitor celecoxib (84), or angiogenesis inhibitor TNP470 (85). These reproducible 24-h variations in drug toxicities have been documented in mice or rats kept in regular alternations of 12 h of light and 12 h of darkness (LD 12:12), a photoperiodic regimen that insures proper interindividual synchronization of circadian physiology, provided that noise, light at night, or external temperature cycles are avoided (86). Similar toxicity rhythms were found for doxorubicin in rodents switched to constant darkness or to constant light, thus ruling out a major masking effect of light or darkness (87). However, animal species,

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strain, gender, and age, as well as fertility and other biological cycles represent additional sources of variability that must be controlled for. Results from experimental chronopharmacology studies have led to studies investigating the relevance of dosing time for drugs in humans. Drug chronopharmacology usually displays reciprocal 24-h patterns in mice or rats and in humans, whose circadian physiology and clock gene expressions differ by nearly 12 h with reference to the light-dark schedule (88). In rodents and humans, the daytime-dependent toxicity and/or efficacy of medications reflect circadian drug uptake and/or metabolism, circadian drug sensitivity of target cells and tissues, or both.

Chronopharmacokinetics

Twenty-four hour changes have been demonstrated for each of the four processes that determine the disposition of more than 300 drugs in rodents and in humans, i.e., absorption, distribution, metabolism, and elimination (ADME) (88, 89). Thus, chronopharmacokinetics determines the exposure patterns of target tissues to the active forms of a drug, irrespective of class, route of administration (oral, transcutaneous or transmucosal (except rectal), intraperitoneal, intravenous), elimination half-life, or acute versus chronic dosing schedule (88–92). However, the physicochemical properties of a medication can influence its oral absorption as well as the extent of dosing time dependency of this parameter (93). Conversely, prominent circadian time dependencies characterize the pharmacokinetics of sustained release preparations at steady state as shown for a theophylline preparation intended for single daily use (**Figure 2*a***) (94). Even during prolonged constant rate infusions of drugs changes in plasma concentrations as large as 50% may be seen over 24 h, as shown for ketoprofen and 5-FU (**Figure 2*b***) (95–97). In the case of a constant rate infusion of a drug with a long half-life, such as vindesine, a cumulative trend is superimposed on the rhythmic circulating drug levels (98).

The 24-h changes in ADME result from the several underlying rhythms that characterize gastric pH; gastric and small intestinal motility; plasma proteins and protein subtypes; membrane microviscosity; limb, liver, and renal blood flows; liver metabolism; and bile volume and salt excretion, as well as renal glomerular filtration rate, tubular reabsorption rate, and urinary output and pH, all part of rhythmic physiology (**Figure 3**). The changes in drug pharmacokinetics over 24 h are still observed in fasting rodents or humans. Yet, concurrent food intake and drug dosing in rodents or in humans usually modify the average pharmacokinetic parameters with little effect on the circadian times associated with peak or trough parameter values (89). The circadian pharmacokinetics of a drug can profoundly impact its tolerability, as previously mentioned for theophyllines (91, 94). For instance, evening dosing resulted in both lowest C_{max} or C_{max}/T_{max} (absorption estimate) and least toxicities for NSAID indomethacin or ketoprofen in osteoarthritic patients (99–102). Such associations between PK parameters and toxicities have been frequently reported in rodent models and in humans. The presence of rhythmic pharmacokinetics calls for the introduction of circadian modulation in the equations that govern drug transfer between compartments to improve the accuracy of parameter estimates, thus

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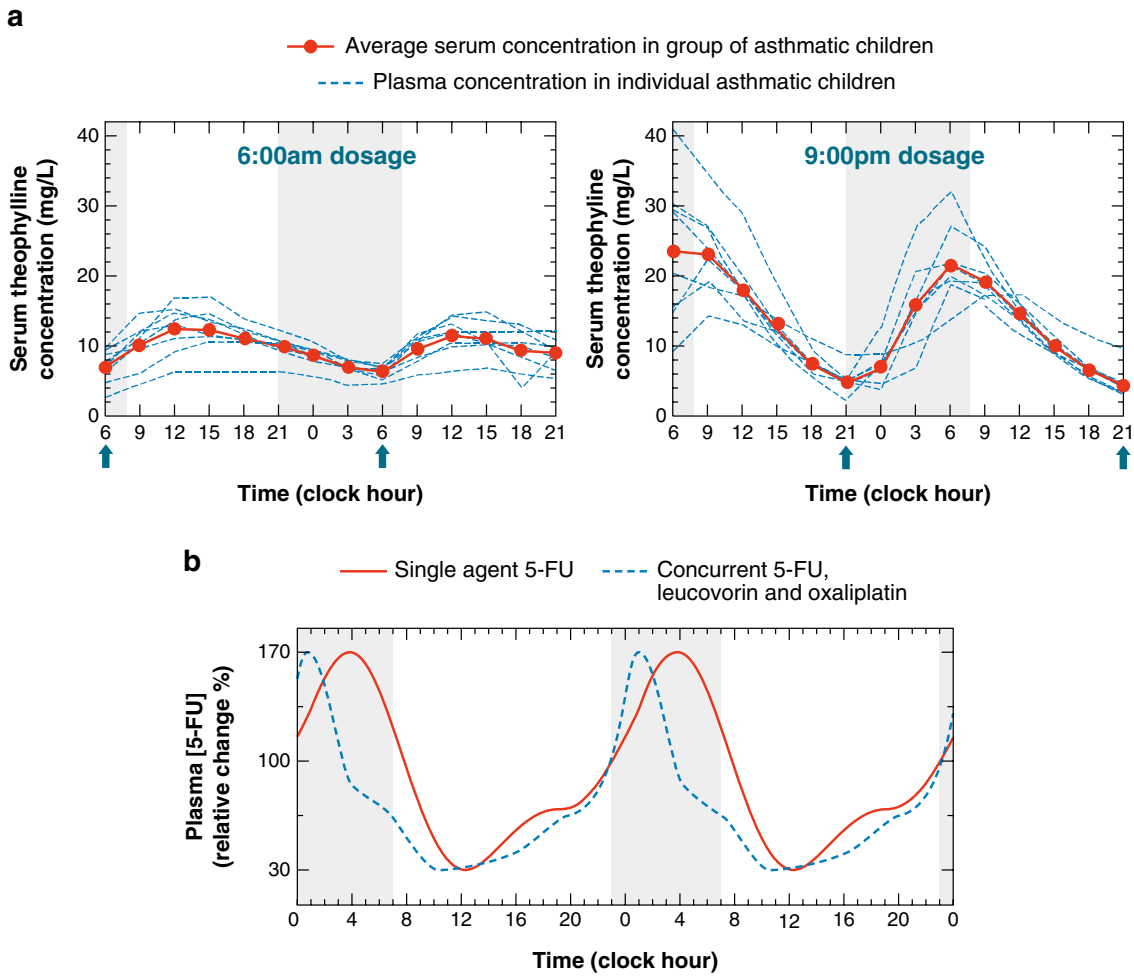

Figure 2

Examples of chronopharmacokinetics despite oral intake of a sustained-release preparation or intravenous infusion at a constant rate for several days. (*a*) Time course of steady-state serum theophylline concentrations in eight asthmatic children receiving Theo24® at 0600 or at 2100 for 6 days prior to study. Results from a double-blind randomized placebo-controlled crossover study. The eight children participating in this study had usual awakening and retiring times of 0630 to 0730 and 2030 to 2200, respectively. The gray area indicates the average sleeping span of the patients. The pharmacokinetic study was initiated at steady state, on the sixth study day. Theo24® was taken following a minimum fasting period of 4 h [i.e., at 0600, 3 h before breakfast served at 0900, or at 2100, 4 h after supper served at 1700 (*arrows*)] (after Reference 94). (*b*) Time course of plasma concentrations of 5-fluorouracil (5-FU) in cancer patients receiving constant rate intravenous infusion for 5 days. The 11 cancer patients participating in both of these studies had usual awakening and retiring times of 0700 and 2200, respectively. Plasma samples were obtained on the first, third, and fifth day of a constant rate infusion of single agent 5-FU (study 1) or concurrent 5-FU, leucovorin, and oxaliplatin (study 2) (after References 96 and 97). For graphical reasons, the 24-h profiles have been duplicated.

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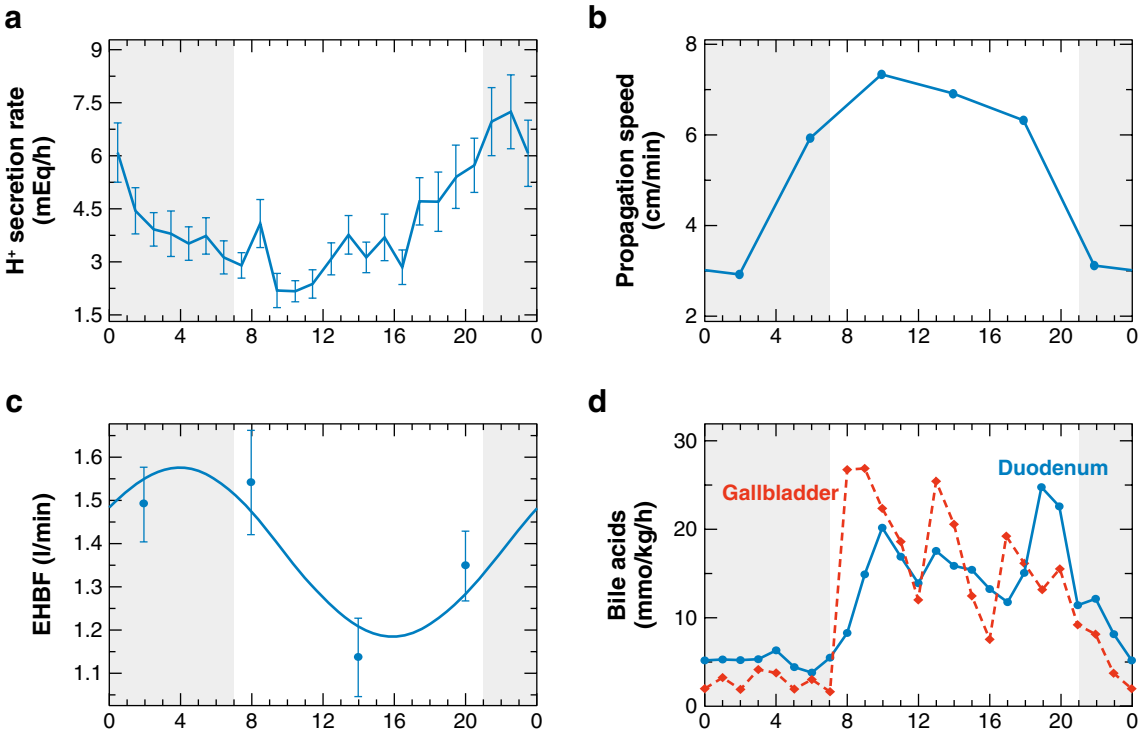

Figure 3

Twenty-four hour changes in selected human physiology determinants of ADME (absorption, distribution, metabolism, and excretion) in healthy subjects. Mean values are shown for each variable considered. (*a*) Basal gastric acid secretion in 14 fasting healthy subjects. Gastric pH can influence drug absorption (after Reference 177). (*b*) Small intestinal motility in 10 healthy subjects (after Reference 178). (*c*) Liver blood flow, as estimated from indocyanin green clearance in 10 supine healthy subjects. This parameter is a major determinant of the metabolic clearance of drugs with high hepatic extraction ratio (after Reference 179). (*d*) Biliary secretion into the gallbladder and bile excretion into the duodenum in five healthy subjects (after Reference 88). (*e*) Plasma concentrations of total proteins and α-1 acid glycoprotein in 14 healthy young adults (after Reference 180). (*f*) Red blood cell membrane microviscosity in seven healthy subjects. Data expressed as relative changes, as percent of individual 24-h mean. Membrane fluidity can influence both cellular drug uptake and receptor availability at the cell surface (after Reference 107). (*g*) Glomeruler filtration rate as estimated with fractionated insulin clearance, urine flow, and urinary excretion of Na+, three determinants of the renal excretion of drugs in healthy subjects (after Reference 181).

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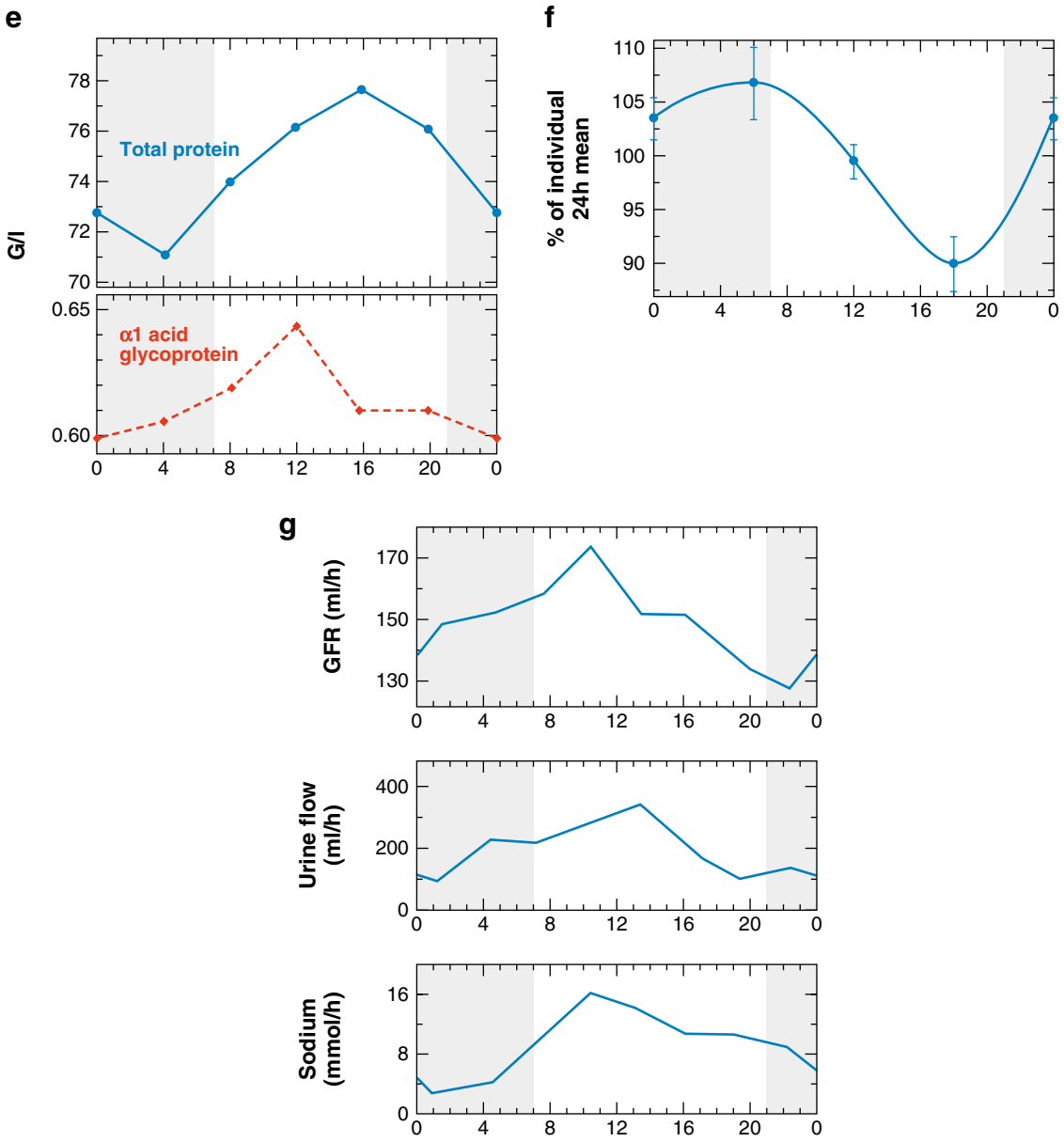

Figure 3

(*Continued*)

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reducing their variability, and to provide important cues for optimal therapeutic dosing time and scheduling. Using such an approach, the antibiotic gentamycin displayed the best renal tolerability following afternoon dosing, a result consistent with the rodent renal chronotoxicity data based on brush border enzymes and urinary release (103–105).

Chronopharmacodynamics

The circadian changes in the interactions between the active forms of the drug and its molecular targets determine the extent of molecular and cellular response. Mechanisms include 24-h circadian rhythms in membrane microviscosity or permeability (106, 107), receptor density or binding enzymatic activities, and transport or repair proteins and ion channels that are known in rodent and in human tissues (74, 108). In vitro exposure of cells to drugs has revealed the role of cellular rhythms as major determinants of pharmacological response. For instance, mouse bone marrow cells were sampled at six different times then cultured in the presence of different concentrations of anticancer drug theprubicin. This study revealed a large-amplitude 24-h rhythm in the relationship between dose exposure and cytotoxic response in vitro. The circadian rhythm in the in vitro toxicity for hematopoietic progenitors matched that of the hematologic toxicity seen after intravenous injection of this drug (109). Similarly, a 24-h rhythm characterized the proliferative response of mouse bone marrow cells as a function of granulo-monocytic colony stimulating factor (GM-CSF) exposure time, whether this agent was delivered at different times to live mice, to fresh bone marrow cells, or to bone marrow cells cultured for up to 4 days (110–112). The recent development of experimental rodent models with mutations in clock genes and/or clock-controlled genes now allow for the molecular dissection of chronopharmacology mechanisms. Thus, a role for clock genes expression in xenobiotic tolerability was recently demonstrated: mice homozygous for an antimorph *Clock* allele or a *Bmal1* null allele displayed increased susceptibility to the anticancer agent cyclophosphamide, whereas $Cry1^{-/-}/Cry2^{-/-}$ mice were more tolerant to this drug. Although cyclophosphamide pharmacokinetics and metabolism differed somewhat as a function of drug dosing or clock gene mutation, the status of the heterodimeric CLOCK:BMAL1 complex in the target hematologic tissue was found to be an important determinant of drug toxicity in wild-type and in clock mutant mice (80). Additional experiments are required to further identify the molecular culprits for circadian cyclophosphamide toxicity and their interacting regulatory pathways.

The main role of cellular rhythms in the elicitation of chronopharmacodynamics is further supported by the demonstration of variable pharmacodynamic effects despite constant rate infusions in patients. Thus, the average anticoagulant effects of heparin nearly doubled between early morning and 0400 in patients with venous thromboembolic disease receiving a constant rate infusion of heparin over 48 h (113, 114). Similarly, the ability of constant rate infusion of famotidine or ranitidine to raise intragastric pH varied threefold along the 24-h timescale despite a constant rate infusion (115).

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Molecular Basis

The genome-wide analysis of transcriptomes in liver has revealed many components involved in xenobiotic detoxification whose expression is circadian at the mRNA level (67, 68, 116). Similar data on the mouse liver circadian proteome have just been reported (72), and the relevance of these molecular bases for chronopharmacology is discussed below.

The detoxification defense system has probably evolved toward the inactivation and elimination of noxious food components. In all mammals, components of the xenobiotic defense system can be classified into three groups (117). Phase I drug-metabolizing enzymes embrace the superfamily of CYP microsomal enzymes. Of nearly 40 known CYP families, 12 have members in all examined mammals. CYPs are expressed at high levels in liver, kidney, intestinal villi, and lung and comprise 150 isoenzymes belonging to 27 distinct genetic families with oxidase, reductase, or hydroxylase activities. Phase II metabolizing or conjugating enzymes comprise sulfotransferases (SULT), UDP-glucuronotransferases (UGT), NAD (P)H:quinine oxidoreductase (NQO), NAD (P):menadione reductase (NMO), epoxide hydrolases (EPH), glutathione S-transferases (GSH), and N-acetyltransferase (NAT). The modification of lipophilic components by these enzymes generally renders these substrates more hydrophilic and thereby increases their excretion into bile and/or urine. Phase III components are transporters, such as P-glycoprotein (P-gp), multidrug resistance-associated proteins (MRP), and organic anionic transporting polypeptide 2 (OATP2). These export-transporters are expressed in many tissues, including liver, kidney, intestine, and brain, in which they serve as barriers against the penetration of endobiotic and xenobiotic components. Aminolevulinic acid synthase (ALAS1) and P450 oxidoreductase (POR) are two additional enzymes playing pivotal roles in detoxification (118). ALAS1 is the rate-limiting enzyme in heme synthesis, and heme is the prosthetic group of all cytochrome P450 monooxygenases. Furthermore, each monooxygenase reaction requires electrons that are extracted from NAD(P)H and transferred to the heme group of cytochrome P450 enzymes via the flavin group of POR. Hence, the expression of CYPs, ALAS1, and POR must be coordinated to permit an efficient detoxification by Class I enzymes. Interestingly, members of Class I, II, and III families, as well as ALAS1 and POR, have been found to display circadian expression at enzymatic, protein, and/or mRNA levels. The times of peaks and troughs of monooxygenase activities in rat liver differed according to the enzyme itself: For instance, aminopyrine N-demethylase was highest by ~30% during the rest span, whereas an opposite rhythm was found for POR (74). These Class I metabolism variations contribute to the daytime-dependent efficacy of drug metabolism and related pharmacologic effects, as shown in a pioneering study on the circadian relation between liver hexobarbital oxydase activity and duration of hexobarbital-induced sleep (119).

The regulation of xenobiotic detoxification is complex, in that the expression of Class I, II, and III components, ALAS1, and POR can be cell-type specific, daytime-dependent, and substrate-inducible. The induction of these enzymes involves several transcription factors that can be considered as xenobiotic sensors. Among them we find the nuclear receptors CAR (constitutive androstane receptor), PXR (pregnane

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X receptor), PPARs (peroxisome proliferator activated receptors, mostly PPARα), LXR receptor (liver X receptor), FXR (farnesoid X receptor), and HNF1 (hepatocyte nuclear factor 1) (120). All of these nuclear receptors have the modular structure typical for steroid receptors and most of them bind their cognate DNA elements as heterodimers with RXR (retinoid X receptor) isoforms. However, other transcriptional regulatory proteins also participate in xenobiotic defense. For example, dioxin receptor (also called aryl hydrocarbon receptor, AhR), a PAS-domain helix-loop-helix transcription factor, induces the expression of the CYP enzyme Cyp1A1 when activated by dioxin and polychlorinated diphenyls (121). Toxic heavy metals, such as cadmium and copper, activate MTF1, a Zn-finger transcription factor controlling the expression of the heavy metal–binding proteins metallothioneins (122). Heat shock transcription factors (HTFs) can also be considered as regulators of the chemical defense system. In fact, these proteins not only induce the expression of chaperones (heat shock proteins) as a consequence of elevated temperature, but also in response to oxidative stress and noxious chemicals (123). Several of the above-mentioned transcription factors either accumulate in a circadian manner, display circadian activity, or are induced in a daytime-dependent manner (71, 124). In the next section, we present a recently discovered molecular pathway that illustrates how circadian oscillators can control the daytime-dependent detoxification of xenobiotics.

Circadian Transcription Factors Control Detoxification Pathways

The proline-acidic amino acid–rich basic leucine zipper (PAR bZip) transcription factor family is composed of the three members: DBP (albumin site D-binding protein), HLF (hepatocyte leukemia factor), and TEF (thyrotroph embryonic factor) (71). These three proteins are among the best-conserved proteins in mammals and show sequence identities between mouse and man of 98%, 96%, and 92% for TEF, HLF, and DBP, respectively. PAR bZip proteins bind DNA elements of the consensus sequence 5′-RTTAYGTAAY-3′ (where R is G or A, and Y is C or T) as homo- or heterodimers (125). All three of these proteins are expressed in a robustly circadian fashion in liver, kidney, and small intestine, three tissues that are particularly relevant for xenobiotic detoxification. The three PAR bZip members also accumulate in most brain regions, albeit with relatively low circadian amplitudes (except in the SCN and the pineal gland) (126). Mice lacking one or two PAR bZip genes only display mild phenotypes, such as sleep disturbances or slight deviations in circadian period length (71, 126). However, mice deficient for all three members suffer from a high juvenile mortality, exhibit a high morbidity at the adult stage, and die prematurely. The high mortality within the first two to three months is caused by spontaneous and sound-induced seizures, probably caused by a deficiency of pyridoxal kinase expression and thus a reduction of the vitamin B6 derivative PLP (pyridoxal phosphate). PLP is a cofactor of many enzymes involved in neurotransmitter homeostasis, and PAR bZip triple knockout mice indeed contain reduced brain levels of serotonin and dopamine. After three months of age, these mice no longer succumb to epileptic seizures, but show signs of accelerated aging, such as cachexia, lordokyphosis, and an absence of vigor. Less than 20% of PAR bZip triple knockout animals reach an age of one year.

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Transcriptome profiling using DNA microarray technology in liver and kidney revealed that PAR bZip transcription factors regulate the expression of many target genes involved in xenobiotic detoxification (71). These include members of all above-mentioned categories of drug-metabolizing enzymes and transporters, as well as ALAS1 and POR, two enzymes required for the activity of all Class I monooxygenases. Moreover, PAR bZip proteins drive the circadian expression of CAR and PPARα, two well-known nuclear receptors involved in the control of drug metabolism. The circadian expression of CAR manifests itself in a strongly daytime-dependent induction of Cyp2B10 mRNA by pentobarbital in liver and small intestine of wild-type animals, whereas the induction is low throughout the day in PAR bZip triple knockout mice. In keeping with these observations, barbiturate-induced sleep duration, which in wild-type mice depends on the time of injection, is constitutively high and dramatically increased in PAR bZip–deficient animals. Moreover, these mutant animals are exquisitely sensitive to the anticancer drugs cyclophosphamide and mitoxantrone.

Par bZip proteins are output mediators of the circadian system rather than core clock components because rhythmic behavior and clock gene expression is not affected in PAR bZip knockout mice (126). Conversely, *Dbp* transcription is driven directly by core components of the circadian clock, such as BMAL1, CLOCK, PER1/2, and CRY1/2 (127, 128). Conceivably, a similar mechanism accounts for the cyclic expression of *Tef* and *Hlf*, although this has not yet been examined in detail. The observations on PAR bZip transcription factors suggest that PAR bZip proteins serve as clock output mediators in the regulation of daytime-dependent detoxification. This clock output circuitry is schematically depicted in **Figure 4**.

CHRONOTHERAPEUTICS

Chronotherapeutics consists of delivery of treatments based on the dynamic changes both in drug pharmacology and in disease-related processes. A first approach to chronotherapeutics is the determination of an optimal dosing time of a drug so that toxicities are reduced and/or efficacy is improved. Thus, glucocorticoids are generally prescribed in the morning, whereas evening dosing is usually recommended for anti-H1 and anti-H2 antihistamines, for several NSAIDs and for several theophylline preparations (24). Rather than addressing the several medical conditions currently involved with chronotherapeutics developments, such as hypertension and cardiac ischemia (90), asthma, cerebrovascular and neurodegenerative diseases, or seasonal depressions (129), we have selected examples of circadian therapeutics in patients with chronic diseases to illustrate the successes, the limitations, and the perspectives of the current approaches.

NSAIDs in Patients with Hip or Knee Osteoarthritis

Pain represents a main symptom that chronically impairs activity and sleep in patients with degenerative joint diseases. Indomethacin and ketoprofen belong to the first generation of NSAIDs used to treat these diseases through inhibition of COX1 and COX2. The main limitations of these medications are gastrointestinal and central

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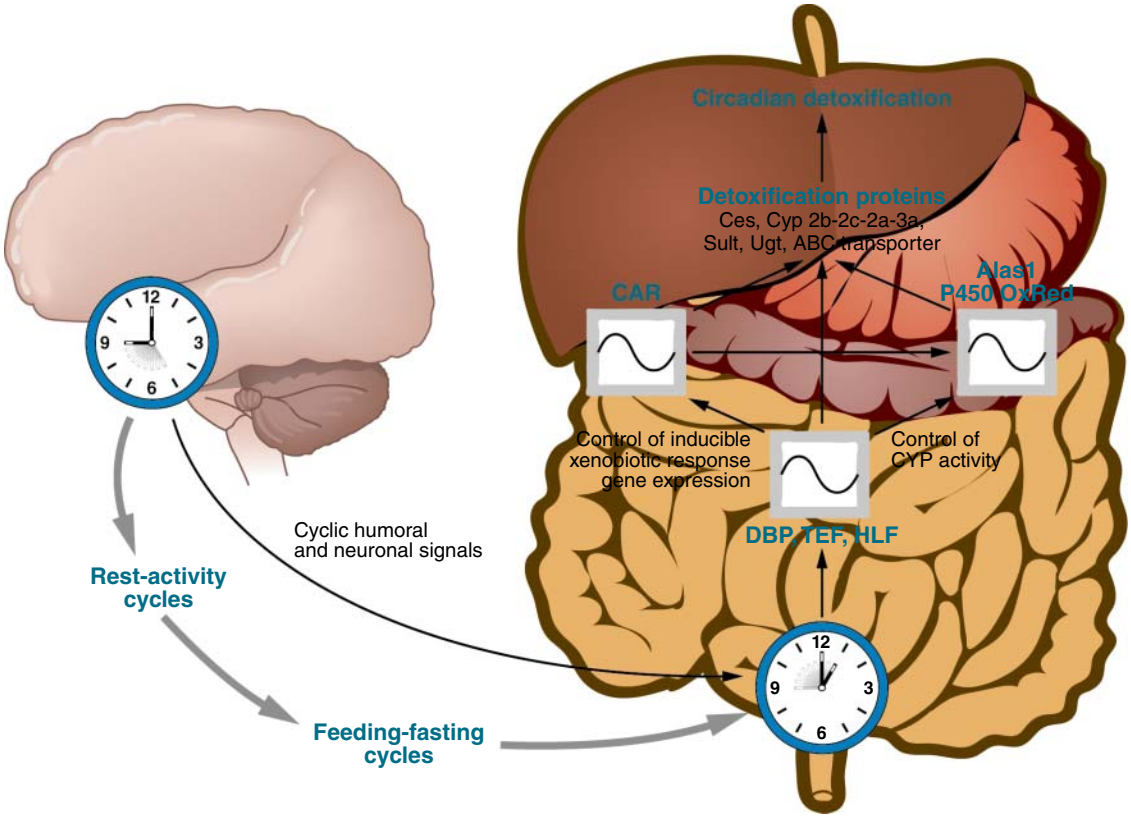

Figure 4

The regulation of xenobiotic detoxification by circadian PAR bZip transcription factors. The SCN master pacemaker synchronizes circadian oscillators in peripheral organs through pathways whose molecular nature is still poorly understood. Because feeding-fasting cycles are dominant zeitgebers for the phase-entrainment of peripheral clocks, hormones associated with energy homeostasis and food metabolites like glucose may play major roles in this process (for review, see Reference 15). The molecular clocks in liver, kidney, and small intestine govern the circadian expression of PAR bZip transcription factors, which in turn modulate the rhythmic expression of regulators and enzymes involved in detoxification (for details see text and Reference 71).

nervous system side effects. Human chronopharmacokinetic studies both in healthy subjects and in patients have shown that morning intake was associated with significantly faster absorption, higher C_{max}, and faster elimination as compared with evening ingestion of indomethacin or ketoprofen [regular or sustained-release (SR)]. Evening dosing of SR indomethacin also resulted in increased hepatic formation of O-desmethyl indomethacin, possibly accounting for the lower C_{max} after evening dosing (100, 130). In clinical trials, SR indomethacin (75 mg) was dosed daily for one week at 0800, for one week at 1300 and for one week at 2000 in randomized sequences to 517 osteoarthritic patients. SR ketoprofen (200 mg) was administered

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daily at 0800 or 2000 in a randomized study involving 118 osteoarthritic patients. The incidence of undesired effects from SR indomethacin was reduced from 33% for morning dosing to 7% for evening dosing (102, 131). Similarly, the incidence of undesired effects from SR ketoprofen was reduced from 47% for morning dosing to 23% for evening ingestion (99). In both trials, treatment withdrawal for toxicity was two- to threefold higher during morning intake. A significant trend toward better pain control in favor of evening dosing was also noticed. However, the baseline circadian pattern of self-rated pain displayed large interpatient variability. Indeed, the individual 24-h variation in joint pain determined the most effective timing of SR indomethacin, found to be at 1300 in the setting of an evening peak and at 2000 in the setting of a flat or bimodal pattern, with an inflammatory component (102). Both of these clinical studies emphasize adequate patient synchronization with regard to the biological mechanisms of NSAIDs chronotolerance, whereas the disease-related determinants of chronoefficacy differed among individual patients. We believe that the clear-cut improvement in NSAID tolerability brought about by evening dosing could be readily considered when attempting to improve the safety of the less-toxic COX-2 inhibitors, such as celecoxib and rofecoxib, given the recent market withdrawal of the latter agent and the experimental chronopharmacology of celecoxib (84).

Cancer

The activity of anticancer agents is limited by their toxicities to healthy host tissues, rendering the determination of the maximum tolerated dose a key step in anticancer drug development. Most chemotherapeutic agents are toxic for cells in the process of cell division, whether these cells are malignant or healthy—the latter predominantly in the bone marrow, gut, oral mucosa, or skin (132). Additionally, many anticancer drugs exert their cytotoxicity at specific phases of the cell division cycle. For instance, cells that are undergoing DNA synthesis (so called S-phase cells) are more susceptible to 5-FU and to irinotecan, two drugs that are widely used to treat gastrointestinal cancers, among several other tumor types, whereas oxaliplatin, which produces DNA cross links and is also active against colorectal cancers, does not appear to have such phase specificity (133–136). Thus, drug metabolism and detoxification, as well as cell cycle–related targets, apoptosis, and DNA repair determine the cytotoxicity of anticancer agents. All these processes are controlled at molecular levels by the circadian timing system. Thus, prominent circadian rhythms characterize both activity and mRNA expression of many enzymes responsible for anticancer drug metabolism in mouse or rat liver. This is notably the case for dehydropyrimidine dehydrogenase (DPD), the rate-limiting enzyme for 5-FU catabolism, as well as thymidilate synthase (TS), a target enzyme for this drug. Similarly, reduced glutathione (GSH) and other thiol groups accumulate in liver and other organs during the activity span of rodents, resulting in circadian cell protection against cisplatin or oxaliplatin damage (137–140). These metabolic processes are well coordinated along the 24-h timescale, so mice best tolerate 5-FU in the early rest span when DPD activity is high and TS activity is low, whereas they best tolerate oxaliplatin near the middle of the activity span of the rest-activity cycle, when GSH levels are increasing (**Figure 5**).

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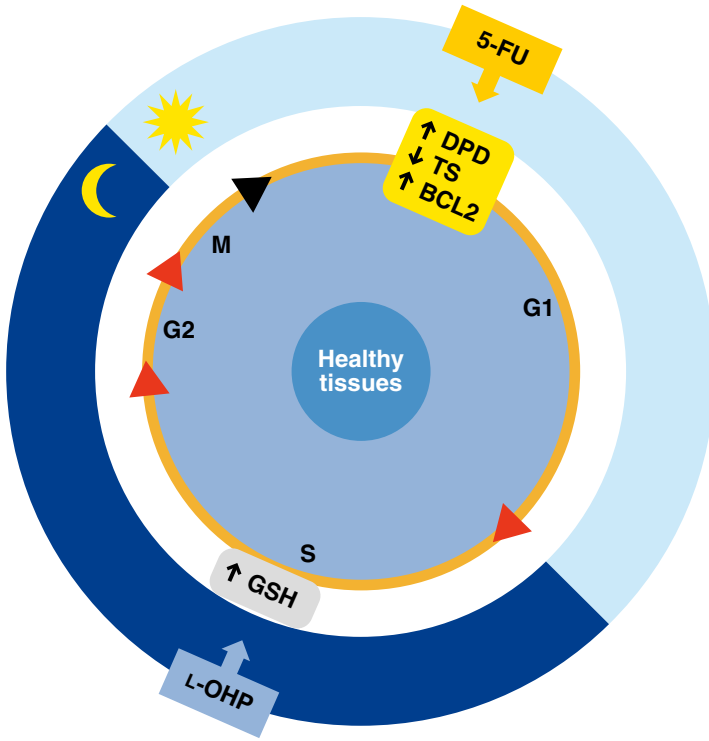

Figure 5

Multiple coordinated mechanisms in the detoxification of anticancer drugs in healthy tissues. Selected example for 5-fluorouracil (5-FU), an antimetabolite, and oxaliplatin, an alkylating agent, in nocturnally active rodents. Following rapid cellular entry, 5-FU is rhythmically catabolized by dehydropyrimidine dehydrogenase (DPD). The active metabolites that are rhythmically formed through pathways not shown here suppress DNA synthesis through inhibition of thymidilate synthase (TS); an enzyme with rhythmic activity. 5-FU can also elicit apoptosis, a process that is antagonized by BCL-2, an antiapoptotic protein that is also rhythmic in bone marrow, a main toxicity target for 5-FU. Finally, proliferating cells are more sensitive to 5-FU following exposure during the DNA synthesis stage (S-phase) as compared to other stages of the cell cycle. After fast cellular uptake, L-OHP interacts with reduced glutathione (GSH) and other thiol-containing peptides and proteins, a process that reduces the formation of DNA crosslinks. GSH and thiol contents in many organs are rhythmic, a process that participates in the detoxification rhythms of many drugs, including oxaliplatin and cisplatin. These cellular rhythms determine a several-fold improvement in tolerability through the delivery of 5-FU during the early light span, when the mice or rats are resting, and L-OHP near the middle of darkness, when the animals are active.

At least two molecular mechanisms further link a molecular clock with the cell division cycle, thus chemosensitivity. The molecular clock controls *Wee1* transcription through an E-box-mediated mechanism (141). WEE1 negatively controls the activity of CDK1/Cyclin B1, which regulates the G2/M transition. In addition, the BMAL1:CLOCK heterodimers repress *c-Myc* transcription through E-box-mediated reactions in the c-Myc gene P1 promoter, and mPER2 can suppress *c-Myc* expression

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indirectly by stimulating *Bmal1* transcription (142). Additionally, antiapoptotic BCL-2 and proapoptotic BAX proteins vary three- to fivefold in mouse bone marrow with peaks occurring during the rest and activity spans of the rest-activity cycle, respectively, a finding in line with the better hematologic tolerability of proapoptotic drugs 5-FU, docetaxel, or irinotecan when dosed during the rest span as compared to 12 h apart (143).

In contrast with the consistent rhythmic changes in drug tolerability mechanisms in host tissues, tumor rhythms appear heterogeneous with regard to clock gene expression and rhythm in pharmacology determinants as a function of tumor type and stage (144–146). This likely results from the cellular heterogeneity of cancers, with frequent mutations, chromosomal deletions, and a hypoxic environment. Possibly corticosterone, as part of host circadian physiology, or exogenous glucocorticoids could play an important role in the synchronization of tumor metabolism and cell division cycle (147). Chronic treatment with prednisolone represses the circadian oscillation of clock gene expression in mouse peripheral tissues (148).

The principles established in preclinical models have guided the development of clinical chronotherapeutic schedules combining 5-FU with oxaliplatin and/or irinotecan against colorectal cancers. In particular, chronotherapeutics was critical in the initial demonstration of the activity of oxaliplatin (Eloxatin®, SANOFI-Recherche), a drug previously rejected for excessive toxicity and poor activity in this disease (149, 150). These multidrug dynamic delivery schedules are administered without hospitalization, at home or during the usual activities of the patient. Thus, a multichannel programmable pump automatically delivers 5-FU-leucovorin (LV, a 5-FU biomodulator) from 2200 to 1000 with peak infusion rate at 0400 and oxaliplatin from 1000 to 2200 with peak infusion rate at 1600 for 5 consecutive days every 3 weeks or 4 consecutive days every 2 weeks (151). At night, compared with daytime, DPD activity is higher in human circulating lymphocytes and fewer cells are in S-phase in human bone marrow, oral mucosa, and skin, which supports a better tolerability of 5-FU (152–154). In the early afternoon, reduced glutathione levels are higher in blood and in human bone marrow, whereas platinum binding to plasma proteins is greater, which supports a better tolerability of oxaliplatin (155–157).

The tolerability, maximum dose intensities, and antitumor activity of these chronotherapy schedules have been evaluated in Phase I, II, and III clinical trials, involving more than 2000 patients with metastatic colorectal cancer (158). In two consecutive randomized multicenter trials involving a total of 278 patients, the chronomodulated regimen combining 5-FU-LV and oxaliplatin achieved 51% objective tumor responses, as compared with 30% in patients receiving constant rate infusion ($p < 0.001$). In addition, chronotherapy reduced the incidence of severe mucositis fivefold, halved the incidence of functional impairment from peripheral sensory neuropathy, and reduced by threefold the incidence of grade 4 toxicity requiring hospitalization, as compared to the flat infusion regimen given over the same duration of 5 days every 3 weeks (158, 159). The relevance of chronotherapy for survival was further investigated in a randomized Phase III trial involving 564 patients in

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10 countries and led by the Chronotherapy Group of the European Organisation for Research and Treatment of Cancer. This study consisted in a pragmatic comparison trial between the chronomodulated infusion of 5-FU-LV and oxaliplatin for 4 days (chronoFLO4) or a sequential 2-day regimen (FOLFOX2) near individual maximum dose intensity every 2 weeks. Both regimens achieved similar median survival, with main dose-limiting toxicities being diarrhea for chronoFLO4 and neutropenia for FOLFOX. The analysis of survival predictors showed that gender was the single most important factor and played a major role for the survival of patients on chronomodulated chemotherapy, with median survival being 16.3 months in women and 21.4 months in men. Indeed, the survival outcome of men was significantly improved on chronoFLO4 as compared to FOLFOX2, whereas the opposite was true for women (160). Mechanisms might involve different (chrono)genotypic profiles between male and female colorectal cancer, as well as gender dependencies in drug chronopharmacology. For instance, the rhythm in 5-FU clearance is marked in men and damped in women (161) and DPD activity is lowest in women, resulting in greater 5-FU toxicity (162). Excessive toxicity can impair the circadian timing system in experimental models (163, 164) and in patients (I. Iurisci, J. Beau, T. Moreau, F. Lévi, "Individual dynamics of circadian rest-activity in patients on chemotherapy," manuscript in preparation), possibly through the release of cytokines and growth factors (165, 166). Among them, TGFα infusion suppresses the rest-activity cycle in rodents (48), whereas interferons and interleukin-6 modify clock gene expression in cellular or rodent models (164, 167) and high circulating levels of both TGFα and IL6 are associated with ablated rest-activity rhythm in patients with colorectal malignancies (168).

Ongoing studies are aimed at confirming the survival benefit in men receiving chronotherapy, as compared to conventional delivery, while developing further optimal scheduling paradigms in women, based on a better understanding of the interactions between the circadian timing system, the cell division cycle, and pharmacology. An interesting finding on this interaction was recently obtained using the cell-dependent kinase (CDK) inhibitor seliciclib in mice with advanced Glasgow osteosarcoma, a tumor with arrhythmic clock gene expression. The extent of tumor growth inhibition varied as a function of dosing time, with a clear induction of near-normal molecular clock in the tumors of mice given seliciclib at the most-effective time, but not at the least-effective time. Such tumor clock induction resulted in enhanced wee1 expression, thus increasing clock-control of G2/M gating, a possible mechanism for slowing down malignant growth. The inhibitory effect of seliciclib on cell cycle progression results from its competitive binding with ATP at the ATP-binding pocket of CDKs. However, seliciclib also inhibits tumor casein kinase Iδ/ε, a central regulator of the circadian clock through the same biochemical mechanism, an effect that could play a central role in tumor clock induction (169). Taken together, these examples reveal that the circadian timing system and its signaling pathways represent potential targets to be shielded or activated in cancer therapy. They further call for improved characterization and monitoring of the circadian timing system dynamics to achieve the largest benefit from circadian treatment scheduling in individual patients.

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THE FUTURE: TOWARD A CUSTOM-TAILORED MEDICINE?

Time Differs Between Individuals

Humans can be grouped into different chronotypes, ranging from larks to owls (170). Patients can also display distinct patterns in circadian physiology, possibly related to the impact of the disease on their circadian timing system components (102, 112, 158). Hence, the physiological significance of daytime differs between human individuals. It is still not clear how much genetics contributes to these different individual behaviors. In the simplest cases, the different circadian phases can be explained simply by period length. Indeed, a modest difference in period length can cause a large difference in phase. Unfortunately, the determination of period length of circadian behavior and physiology in human individuals is costly and time consuming, and thus cannot be done on a routine basis in the clinic. However, the recording of period length can be accomplished relatively easily on cultured primary fibroblasts obtained from skin punch biopsies, using lentiviral vectors encompassing a circadian luciferase reporter gene (171). Such experiments revealed an unexpectedly high variability between human individuals. Although it is not yet clear whether fibroblast gene expression cycles correlate with human circadian physiology, similar experiments with mice carrying mutations in clock genes suggest that at least qualitatively, fibroblast rhythms correlate with locomotor activity rhythms (171, 172). In isolated cases, circadian periods could also be monitored in primary keratinocytes from plucked hairs using lentiviral vector technology (171). Plucking hairs is obviously less invasive than skin punch biopsies, and it is hoped that the keratinocyte procedure can be optimized to make it generally applicable. If these attempts succeed, it should become possible to record circadian gene expression cycles for hundreds to thousands of human individuals.

With the advent of new technologies permitting the rapid establishment of high-resolution haplotype maps (173), the high-throughput determination of period length may reveal new quantitative trait loci contributing to individual differences in human circadian biology. In turn, the knowledge gained from such studies may be applicable in chronotherapeutic approaches in the clinic.

Human Haplotype Genetics and Personalized Chronopharmacology

Attempts to personalize chronotherapy obviously require profound knowledge of both the circadian timing system and the pharmacogenomics profile of a patient.

Genomic data acquired through the international HapMap project will contribute tremendously to these efforts (173). As of October 2005, nearly 10^7 nonredundant SNPs have been reported for the genomes of three ethnic groups (90 individuals each of Yorubans, Europeans, and East Asians). Most haplotypes are represented by a small number of ancient versions dating back to the time before the European

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population separated from the original African population. Thus, it was estimated that approximately 80% of the human genome is covered by only three haplotypes (174). Genomic diversity is caused mostly by two factors, the mutation rate per generation, which probably has not changed much in the past, and the size of the population, which has exploded during the past 100,000 years from approximately 10^5 to 7×10^9 individuals. In other words, the number of polymorphisms is far from that expected at equilibrium, and most of our haplotypes still correspond to the ones present before the exponential growth of the human population. The fixation of alleles in the population has a complex basis, and, apart from population size and mutation rate, is influenced by positive and negative selection. In particular, the genes relevant for detoxification (and hence pharmacology), are likely to be under positive and negative selection in populations with different diets and lifestyles. This is beautifully illustrated by a recent report in which relatively recent alleles that have not yet reached fixation were mapped in the genomes of three ethnic groups with different lifestyles (sub-Saharan Yorubans, East Asians, and Europeans) (175). Indeed, some Phase I and Phase II drug-metabolizing enzymes are among the genes that are under different selection pressure in the three examined cohorts. More focused genetic attempts are also underway in which polymorphisms in genes relevant for specific drug targets are investigated. This has resulted in a database of polymorphisms in 267 drug-related genes, encompassing Phase I and Phase II metabolizing genes as well as Phase III transporters (176). Given the strong daytime-dependence of drug metabolism in animal and human systems, circadian biology should be taken into consideration when examining the impact of such polymorphisms on drug efficacy and drug toxicity. There are and must be ethical concerns related to the geno- and phenotyping of human individuals, and all efforts must be undertaken to guarantee the privacy of such information. If these ethical issues can be handled appropriately, we feel confident that the genetic information gained on the circadian timing system and the fine structure of our haplotypes combined with lifestyle and circadian physiology monitoring through noninvasive technologies will open promising new avenues toward custom-tailored chronotherapeutics.

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