Title

The Dosing-Time Makes the Poison: Circadian Regulation of Pharmacotherapy and Pharmacotherapy of Circadian Clocks

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Abstract

Daily rhythms in physiology significantly modulate drug pharmacokinetics and pharmacodynamics according to the time-of-day, a finding that led to the concept of chronopharmacology. The importance of biological clocks for xenobiotic metabolism has gained increased attention with the discovery of the molecular circadian clockwork. Mechanistic understanding of the cell-autonomous molecular circadian oscillator and the circadian timing system as a whole has opened new conceptual and methodological lines of investigation to understand (1) the clock's impact on daily variations of a specific drug's or environmental substance's effects and/or side-effects, and (2) how clock-controlled pathways are coordinated within a given tissue or the whole body.

Today, there is an increased understanding of the circadian modulation of drug effects. Thus, the circadian coordination of Phase I, II and III xenobiotic metabolism can be viewed as an adaptive and anticipatory time mechanism, which most efficiently help increase xenobiotic water solubility and excretion. Interestingly, the circadian clock itself has been identified as a target for pharmacotherapy. Indeed, several molecular strategies are being developed to treat disease-dependent and drug-induced clock disruptions in humans.

The Role of the Circadian Timing System in Xenobiotics detoxification

Recent scientific evidence highlights the critical role of circadian rhythms for the metabolism and effects of **xenobiotics** (see Glossary), including drugs as well as environmental toxicants (Figure 1). Since 2011, there has been increased awareness on the regulation of circadian rhythms in pharmacology or toxicology. Conceptual and methodological progress has enabled the tracking of circadian patterns in cells, tissues, experimental animals and human beings [1-6]. These new insights have improved our understanding of the underlying molecular mechanisms and systems level organisation of the regulatory circuits, which modulate cellular metabolism and proliferation during the course of a 24 h day [7-9].

It has long been known that the **Circadian Timing System (CTS)** accounts for time-varying effects of xenobiotics with up to 10-fold magnitude, according to the timing of exposure, supporting the need for an increased understanding of chronopharmacology and chronotoxicology [10-12]. Highly reproducible 24 h variation in drug toxicities has been documented in mice or rats kept in regular alternations of 12 h of light and 12 h of darkness (LD 12:12), as well as in constant darkness, thus unmasking possible direct effects of light on various endogenous and metabolic rhythms, e.g. cortisol [13]. However, animal species, strain, sex, age, fertility, as well as yearly and other biological cycles can represent additional sources of variability. Results from experimental chronopharmacology studies have led to investigate the relevance of time of dosing on the effects drugs or treatments may have in humans. Drug chronopharmacology usually displays opposite 24 h patterns in nocturnal rodents when compared to people, whose circadian physiology and

molecular clock gene expression differ by nearly 12 h relative to the light-dark schedule [14]. Recent experimental data using targeted anticancer agents have further determined that both circadian timing and drug dose play important roles in the determination of systemic exposures and therefore, of the pharmacologic effects (Table 1). Clinical trials including randomized Phase III studies or meta-analyses of chronotherapy schedules have resulted in up to five-fold better drug tolerability and a doubling in drug efficacy as compared to conventional non-time-stipulated treatment schedules [15](Table 2). By contrast, a number of randomized comparisons between morning and evening dosing times have demonstrated similar rates of adverse events and/or efficacy for several medications [16-19]. This suggests that either the optimal timing was missed in the study design, excessive dose levels were tested, or differences between patients led to an underestimation of the timing effect. Indeed, experimental and clinical data have revealed broad inter-individual CTS differences, resulting in different chronotoxicity profiles [6, 20]. Such differences can result from genetically-determined "**chronotypes**" as well as from epigenetic changes, age, sex, lifestyle, disease or pharmacological treatment [21-24]. Moreover, circadian disruption has emerged as a novel concept, identifying a lack of proper coordination between different components of the CTS as a contributing factor for developing cancer, metabolic syndrome and cardiovascular or infectious diseases [25-29]. Circadian disruption has further been associated with occupational shift-work [30]. It has also been linked to poor disease outcomes, especially in cancer patients [31]. The identification of subjects with different, yet functional CTS, and subjects with disrupted circadian rhythms have fostered the idea that circadian clocks could be therapeutically targeted [32]. This review focuses on the recent progress that has been made in identifying the mechanisms underlying the interactions between the CTS, disease and pharmacotherapy.

Dosing-time Dependencies of Xenobiotic Effects

Drug development aims to define a recommended dose for a potential new compound based on the majority of individual subjects, irrespective of timing, sex, age, lifestyle or comorbidities. However, unanticipated or overwhelming

adverse events represent severe limitations, resulting in both drug attrition [33, 34] and post-marketing withdrawal of several otherwise effective medications [35, 36]. Moreover, some countries, such as the UK, are now terminating the reimbursement of several medications (including anticancer drugs), despite demonstrated efficacy and safety in randomized Phase III trials, or their approved use by American, European, and other national/international regulatory authorities [37, 38]. An alleged rationale is that the toxicities of these new agents sometimes outweigh the slight benefits in efficacy at a population level, thus making these new treatments too costly for the healthcare system. As a result, medication safety represents a crucial challenge that needs to be prioritized and addressed with new concepts and methods at all stages of drug development and post-market approval. A large body of evidence, from mice to patients, supports the notion that chronopharmacology could indeed help minimize adverse events through the identification of optimally-timed drug delivery. An additional concern is the impact of circadian disruption (as observed in occupational shift-work), on an organism's response to and detoxification of environmental xenobiotics.

In past decades, the majority of chronopharmacology and chronotoxicology research has focused on the determination of xenobiotic exposure times leading to either highest or lowest toxicity or efficacy in rodents [10, 11]. Chronotoxicology or chronopharmacology measures have been established for many substances and marketed drugs in laboratory animals and/or human beings. Human chronopharmacology studies have further established dosing time dependencies for over 300 medications of all classes, including clinical validation of timing effects in randomized Phase III trials for a few of them [15, 39](Table 2). Moreover, an improved mechanistic understanding of the CTS in addition to obtaining better tools for continuously monitoring the CTS at the molecular level and in real-time, provides proof-of-principle data for *in vitro* chronopharmacology testing (see below).

The circadian timing system (CTS)

The generation of circadian oscillations has been shown to occur at the level of the single cell. The molecular mechanism of this cell-autonomous

transcriptional/translational feedback loop has been largely elucidated, although further levels of control are still being discovered (Text Box 1). In multi-cellular organisms, all of these individual cellular clocks are coordinated by a central pacemaker that receives environmental light input and feedback from peripheral oscillators. In mammals, the suprachiasmatic nuclei (SCN) of the hypothalamus have been identified as this central pacemaker [40], which orchestrates behavioural and physiological rhythms like rest/activity, body temperature, and hormonal patterns, such as the 24-h cortisol rhythm in human subjects [41]. The inputs and feedbacks that provide time cues to the SCN are mediated through a variety of neuropeptides or direct axonal contact [41].

Elucidating the dynamic relative contributions of **peripheral and central clocks** in physiology and pathophysiological alterations has only been feasible through the use of in vivo real-time bioluminescence recording in freely moving mice [4]. Most likely, multiple other processes are involved in the synchronisation of peripheral clocks. Even in the absence of the SCN or a tissue-intrinsic clock, some metabolic or proliferation pathways are still "driven" by these yet unknown signals, and/or an organism's surrounding rhythmic physiology [4, 42, 43].

Depending on the adaptation of a species, feedback signals from the periphery may have variable effects on the SCN. Some **non-photic signals** such as physical activity have great impact on the central pacemaker in specific rodent models [44], and possibly in human individuals, too. However, non-photic signals compete with light signals in terms of adaptation [45-47]. Most species show entrainment to food as a time cue. In mice, the liver has been shown to reset its clock based on the timing of food intake, independent of SCN signalling [48, 49]. Of note, food anticipatory activity is SCN independent in rodents and remains intact in BMAL1 but not PER2 deficient mice [50, 51]. Interestingly, recent evidence suggests that it is hepatic *Per2* regulated b-hydroxybutyrate production dependent [52].

Importantly, food availability has also been shown to compete with light-dependent signals coming from the SCN, and can lead to a situation where the SCN and liver clocks are uncoupled from each other [4, 53]. Such

uncoupling due to mistimed sleep has also been found in mouse under simulated “shift-work schedules”, and was suggested to be associated with metabolic disruption [54]. In fact, mistimed food intake has been shown to lead to obesity and metabolic syndrome in mice and human subjects [55-57].

Critical importance of peripheral clocks

A developing and important question about peripheral clocks is what their impact on physiological processes is and therefore, also on what their role is in the modulation of pharmacotherapy. For example, the phase of the liver intrinsic clock is important for drug metabolism. For instance, the rhythm in acetaminophen toxicity with high toxicity during the night but low toxicity during the day is critically dependent on the hepatocyte circadian clock. Mice with liver-specific ablation of BMAL1 or CLOCK lack a rhythm in acetaminophen liver toxicity [58, 59]. Daytime feeding inverts this rhythm in nocturnal rodents, which mostly feed during the night phase, leading to high toxicity of acetaminophen during the day [60]. This illustrates that if peripheral tissue-intrinsic clocks regulate key steps of a molecular pathway, the deregulation of tissue clocks might represent an important pathological focus and lead to new potential pharmacotherapeutics.

Furthermore, peripheral tissue clocks have been shown to be essential for proper physiological function in mice; even if all other peripheral clocks and the central pacemaker are intact. Most of this work has been carried out by selectively deleting clock function in specific organs or cell populations. For instance, genetic ablation of the circadian clock in pancreatic beta cells-specific BMAL1 deficient mice has been shown to lead to type II diabetes [61, 62]. Similarly, cardiac functions like myocardial contractility are impaired in *α-myosin heavy chain-Clock*$^{\Delta 19}$ knock in mice without a functional clock in cardiomyocytes [63]. *Krüppel-like factor 15* (*Klf15*) is thought to link the circadian oscillator to the regulation of cardiac potassium channels important for cardiac repolarisation, and therefore ventricular arrhythmias in mice because cardiomyocyte-specific *Klf15* deficient or overexpressing mice do not show circadian QT interval regulation [64]. Even the local disruption of peripheral clocks in the brain has important implications for the whole

organism. Deleting the circadian clock mechanism in histaminergic neuron populations in mice by locally deleting BMAL1 expression has been shown to alter histamine brain levels and consequently lead to sleep fragmentation and shallower NREM sleep [65]. Cell-intrinsic clocks in various immune cell populations have been shown to be of functional importance for time-of-day variations in both innate and adaptive immune functions [66-69]. Most recently, the circadian clock in pulmonary epithelial club cells was found to modulate recruitment of neutrophils to the lungs in response to a bacterial challenge. In wild-type mice circadian expression of the chemokine *Cxcl5* in club cells and systemic glucocorticoid levels modulate neutrophil recruitment. In mice with BMAL1 deficient bronchiole cells, however, constant CXCL5 increases inflammatory responses after bacterial challenges, despite persistent circadian glucocorticoid rhythms [68]. Of note, simulated shift-work in human volunteers disrupts the coupling between rhythms in cytokine secretion and relative abundance of monocytes and T-lymphocytes [70].

Such non-exhaustive list of examples illustrates the functional importance of tissue intrinsic clocks and emphasizes the potential impact of circadian disruption. It remains to be seen, however, if rescuing or pharmacologically enhancing rhythmicity in peripheral clocks could become a relevant treatment option in chronic diseases.

Interactions between the circadian clock and the cell-cycle

Possible consequences of clock disruption include a higher incidence of cancer and accelerated cancer progression. In experimental cancer models, SCN ablation or simulated shift-work schedules have been shown to accelerate tumor growth [25, 71]. In patients, epidemiological evidence suggests that shift workers have higher cancer incidences and breast cancer patients with misaligned sleep tend to have shorter disease free survival [72-74]. This reflects in part the tight link between cell cycle and the circadian clock. The cell cycle has long been known to be synchronized by the CTS in mammals [75]. Twenty-four-hour rhythms have been demonstrated in DNA synthesis and mitotic activity *in vitro* in many cells and *in vivo* in many rodent and human tissues [9, 76-78]. Moreover, circadian synchronized cell cycling

has been recognized as an important mechanism accounting for the chronotoxicity of some anticancer drugs, such as gemcitabine, irinotecan, 5-fluorouracil or docetaxel [10, 79]. Based on studies in mouse liver and in cultured fibroblasts, a gating mechanism controlling the G2/M transition via CLOCK/BMAL1-activated WEE1 kinase was initially considered. Subsequent studies have suggested further mechanisms by which the clock and the cell cycle are coupled [80-83]. As such, a common theme emerges: the circadian clock controls the expression of several cell cycle-related genes, which in turn modulate the expression of key regulators of mitosis. The combination of long-term clock and cell cycle reporter recording at the single cell level has further shown, using mathematical modelling, that the circadian clock and the cell cycle should be considered coupled oscillators, with reciprocal interactions [1, 5]. This suggests that the clock can control cellular proliferation, but also that cellular proliferation can influence the clock. This relationship could further represent a critical determinant for the time-dependencies of the cell cycle effects of many drugs and environmental toxicants. However, whether such coupling also exists *in vivo*, displays any tissue specificity, or is altered in proliferative diseases remains unknown as yet.

Role of circadian clocks in pharmacology and toxicology

Twenty-four-hour rhythms have long been known to moderate xenobiotic absorption, distribution, metabolism and excretion. These key processes determine the shape and levels of cellular exposure to drugs and toxicants, i.e., pharmacokinetics and toxicokinetics [10]. An epidemiologic study involving 14480 patients with intentional self-poisoning (oleander seed or organophosphorus) further highlights the tight links between the time of poisoning and death in the human population. Up to 50% reduction in case fatalities were observed if evening rather than late morning poisoning occurred; a difference that does not seem to be explained by the treatment but was suggested to be influenced by intestinal P-glycoprotein (P-gp) and hepatic cytochrome P-450 (CYP) 3A4 rhythms [84].

There are recent advances in the understanding of the phases of circadian control of xenobiotic metabolism, namely, Phase I, oxidation, reduction and hydrolysis reactions; Phase II, conjugation reactions; and Phase III, xenobiotic transport. These processes have been shown to ultimately increase xenobiotic water solubility and excretion mainly via urine and bile [10, 79].

Phase I and II metabolism in mouse liver, kidney and intestine have been shown to be regulated through rhythmic expression of E-box dependent proline and acidic amino acid-rich basic leucine zipper transcription factors (PARbZip) [85]. PARbZip transcription factors bind rhythmically to D-box containing promoters of key genes that regulate xenobiotic metabolism, such as cytochrome P450 oxireductase (POR), constitutive androstane receptor (CAR), peroxisome proliferator activated receptor-α (PPAR-α), and aryl-hydrocarbon receptor (AhR) [85]. Moreover, microsomal and non-microsomal oxido-reductases and esterases also display circadian rhythms not only in mRNA and protein, but also at the enzymatic activity level. As mentioned above, circadian modulation of CYP activity results in dosing time and functional hepatic clock-dependent differences in acetaminophen toxicity in mice [58, 59]. Acetaminophen is metabolized by CYP3A4, and human CYP3A4 is important for the biotransformation of half of all marketed drugs. Indeed, in healthy human subjects, CYP3A4-dependent metabolism of the benzodiazepine derived anxiolytic midazolam is 20% higher in the middle of the day when compared to the middle of the night [86].

Carboxylesterases (CES) also play a pivotal role in Phase I metabolism and are under direct transcription control of PARbZip proteins as has been found *in vitro* [87] and *in vivo* [85]. Indeed, the rhythmic control of CES has been shown to be important for the circadian bioactivation of anticancer agents such as irinotecan and capecitabine [79]. Another important Phase I enzyme, dihydropyrimidine dehydrogenase (DPYD) is circadian regulated, resulting in time-dependent dehydrogenation and deamination of fluoropyrimidine drugs, such as fluorouracil and capecitabine [79], respectively.

With regard to Phase II drug metabolism, the circadian rhythms of glutathione S-transferase (GST) activity and glutathione (GSH) content have been reported to be highly important for the detoxification of xenobiotics as is the

case for instance, of acetaminophen [60], or metal compounds such as cadmium [88] or platinum complexes. In rodents, the GSH contents in the liver and jejunum are approximately threefold higher during the second half of the night when compared to mid-day [89]. In support of this, PARbZip-deficient mice exhibit a general down-regulation of *Gstt1* and *Gsta3* gene expression and are subsequently less susceptible to acetaminophen toxicity [85].

Following solubilisation, Phase III transport of compounds in the liver, kidney and intestine are mainly accomplished by ATP binding cassette (ABC) transporters [90]. Many ABC transporters including *abcb1a, abcb1b* (the rodent homologs of P-gp) and other ABC members *abcc2* and *abcg2* have been shown to exhibit circadian expression patterns in the intestine and liver in rodent models [91-98]. Transcriptional rhythms have also been demonstrated to lead to higher daytime P-gp activity in the jejunum and ileum of rats [99]. Solute carrier (SLC) superfamily transporters, are mainly responsible for drug influx into the intestine, liver, kidneys [90]. In mice, hepatic circadian expression patterns have been observed in various organic anion transporting polypeptides, the *organic anion transporter-1 (Oct1)and 2* (*Oct2*)[92]. In addition, rhythmic PPAR-α driven OCT2 protein abundance has been implicated as an important regulator of the circadian rhythm of cisplatin nephrotoxicity in mice [100].

The daily variation of enzyme and transporter activity involved in the metabolism of a given substance is a striking observation. For instance, both *in vitro* and *in vivo*, the maximum in the bioactivation of irinotecan by CES occurs near the nadir in its detoxification enzyme UGT1A, and vice versa [101]. In aggregate, the circadian coordination of Phase I, II and III xenobiotic metabolism and transport pathways represents an anticipatory timing mechanism that most efficiently helps to increase xenobiotic water solubility and excretion [79]. Such endogenous circadian organisation likely reflects the adaptation of living beings to environmental 24 h cycles in possible xenobiotic exposure.

A New Way Forward: *In Vitro-In Silico* Circadian Modulation of Xenobiotics Effects

The tight coordination of metabolic pathways across the day shows strong inter-individual variance, but also can be altered, in particular whenever pathologic processes or treatments disrupt the CTS. Therefore, there is a need for a systems approach to chronopharmacology in order to systematically map the key clock-controlled metabolic processes and test the consequences of their alterations on chronopharmacology. Expectedly, such systems chronopharmacology will help make *a priori,* predictions of the specific chronopharmacology pattern of a given substance, according to an individual's CTS as assessed by one or more suitable biomarkers. A first step toward this new strategy has been to combine *in vitro and in silico* investigations. For instance, in contrast to chronopharmacology or chronotoxicology studies *in vivo*, investigations in circadian synchronized cell culture models presently allow systematic and quantitative testing of drug compounds, subsequently generating mathematical models to quantify the impact of molecular clocks on xenobiotic metabolism (24). An example is the *in vitro–in silico* circadian investigation of the cancer chemotherapeutic irinotecan pharmacokinetics-pharmacodynamics, which were performed in differentiated human epithelial colorectal adenocarcinoma (Caco-2) cells [87, 101]. Results showed that transcriptional rhythms were observed in all phases of irinotecan metabolism: Phase I (CES), II (UGT1A1) and III (ABCB1) [101]. For example, the CES-mediated biotransformation of irinotecan into its active metabolite SN38, doubled depending on the circadian phase cells were exposed to irinotecan. All these effects taken together, this led to a 4-fold change of irinotecan-induced apoptosis depending on the timing of drug exposure. When the circadian clock was disrupted by siRNA mediated *Bmal1* silencing, however, drug timing dependent rhythms of drug metabolism and apoptosis were absent [101]. These findings illustrate how *in vitro* chronopharmacology and chronotoxicology might contribute to a cost-effective optimisation of preclinical drug development and/or toxicant testing.

Each on their own, different *in vitro* systems might reveal further differences in circadian dynamics of drug metabolism, potentially revealing cell or tissue specificity and proliferation status or inter-individual differences irrespective of

comparable molecular clock proficiency. For example, by contrast to Caco-2 cells, clock-containing proliferating Glasgow osteosarcoma cells did not exhibit a circadian pattern in *abcb1a* or *abcb1b* gene expression [102].

Usefulness of Circadian "Omics"

The *in vivo* and *in vitro* drug metabolism circadian investigation approaches might indeed benefit from "omics" technologies. Multiple pharmacology and toxicology studies have shown that circadian clocks regulate key molecular pathways of drug metabolism in animal models. For studies of liver drug metabolism, various recent transcriptomic, proteomic and metabolomic circadian data-sets are now available from mice [103-107]. This has been extremely useful for systems biology approaches to drug metabolism. However, fewer time-series studies have been published in other putative drug target tissues such as the heart and aorta [27, 108, 109], the kidney [110] or the central pacemaker, the SCN [111]. Comparing circadian patterns of multiple tissues is especially interesting and informative because it casts insight into tissue-specific clock-controlled mechanisms of xenobiotic metabolism. For example, circadian expression profiles of more than half of all nuclear receptor genes, (which represent important metabolic sensors in key tissues such as the liver, skeletal muscle and fat) have established a clear tissue-specific circadian regulation of energy metabolism in mice [112]. However, only one drug metabolism study has been conducted to compare circadian gene expression in a dozen mouse tissues [106]. The resulting data suggest that many disease-relevant genes operate under the control of circadian clocks, but also, that many drug targets are circadian genes themselves. In fact, a large portion of marketed drugs has effects on circadian genes, with a half-life of less than 6 h [106]. This could be conducive for the development of chronotherapeutic approaches for these compounds. Key drug metabolism pathways have important roles in extra-hepatic functions. For example, the human CYP-P450 system, contributes to the bioactivation of multiple drugs in intestinal and respiratory tissues, and is highly regulated by molecular clocks with tissue-dependent phases of gene expression [113]. Of note, circadian metabolomics have been recently described in various biological samples such as blood, saliva, urine and exhaled breath [114-119].

These matrices might offer great translational potential as biomarkers for the clinic because they are available from animals and human subjects alike [114-117, 120], and facilitating non-invasive repeat sampling [118, 119], in time-series or “round-the-clock” data-set collection.

Effects of Drugs on the CTS

The CTS modulates drug pharmacology and toxicology through a multitude of processes. There is growing evidence of the effects of drugs on the CTS, as shown by the circadian disruption of rest-activity, body temperature or clock gene expression patterns. In mice and human patients receiving chemotherapeutic drugs severe alterations of physiological rhythms have been observed [6, 121, 122]. Broadly, these drugs can be grouped into (i) those exhibiting unintentional side-effects or unspecific toxicity, resetting the clock, and (ii) targeted chronodrugs. With a promising outlook, new agents such as RevErbα agonists [123] are currently being developed to target either CTS coordination or the molecular clock for different tissues in order to enhance the robustness of these components and/or modify circadian phases. Moreover, mathematical modelling of core clock genes *Bmal1* and *RevErbα* expression patterns in mouse liver or colon, have proved to be predictive of different chronotoxicity patterns for the drug irinotecan [20].

Effects of Xenobiotics on the CTS

Chemotherapeutic drugs in particular have been described to have resetting and dampening effects on circadian oscillations. These agents can also unintentionally modify the CTS by either disrupting CTS coordination, or by altering **circadian amplitude or phase**. As such, the CTS can represent a toxicity target worth to be shielded through proper circadian drug timing. Indeed, certain indicators of CTS coordination such as rest/activity and core body temperature can be severely disrupted by anticancer agents of any pharmacologic class in mice or rats [reviewed in 79]. Twelve anticancer agents including cisplatin, carboplatin, oxaliplatin, 5-fluorouracil, irinotecan, seliciclib, and everolimus among others, have been shown to impair molecular circadian clocks in the SCN, liver, adrenals, and other peripheral organs of

mice and in cell cultures [79]. Moreover, the extent of the alterations and the recovery dynamics of rest-activity and body temperature rhythms depend on dose as well as on circadian timing [124]. Thus, inappropriately timed anticancer agents are capable of modifying circadian clock amplitude and phase in peripheral organs, preventing the predictability of internal circadian timing. Indeed, the clinical relevance of treatment-induced circadian disruption has been demonstrated in cancer patients receiving chemotherapy, and using the rest-activity rhythm as a CTS “biomarker” [122, 125].

Chronodrugs – Clocks as Targets

The hidden resetting of clocks by drugs presents a problem in terms of proper timing for repeated daily dosing. Interestingly, the dosing-time dependent toxicity persists or is even amplified during the chronic dosing of anticancer agents such as taxane derived docetaxel, the alkylators carboplatin and oxaliplatin, or the cyclin-dependent kinase inhibitor seliciclib [79]. This finding is in line with the dosing time dependency of drug-induced circadian disruption. However, targeting specific agents at the CTS might counteract the disruptive effects of some drugs through purposely resetting circadian rhythms to a specific phase and/or by enhancing their amplitudes. Such is the case for drugs that act on the neuronal network of the master pacemaker in the SCN. The SCN is reset by guanylyl cyclase-cGMP-protein kinase G dependent mechanisms, which have been described more than a decade ago [126]. More recently, this pathway has been exploited using “sub-erectile” doses of the cGMP-specific phosphodiesterase type-5 (PDE5) inhibitor sildenafil to alleviate jet-lag and shorten physiological adaptations following trans-median travel [127]. Similarly, it has been suggested that faster circadian resetting could result from pharmacological uncoupling of the SCN neuronal network. Desynchronised SCN neurons because of their smaller combined amplitude would then be more easily reset to the new phase [128].

Even more classical, yet mechanistically not fully understood, the pineal hormone melatonin is known to reset the circadian clock. Recently, melatoninergic agent like ramelteon and combined melatoninergic/serotonergic drugs like agomelatine have become available in

the clinic to treat insomnia and depression, respectively. Like melatonin, their mechanism of action might involve a resetting effect on the SCN clock mediated by the melatonin receptors MT1 and MT2 [129, 130]. Another example is lithium, a marketed drug used in the treatment of bipolar disorders that has been shown to lengthen the period of the circadian clock. Most likely through inhibition of glycogen synthase kinase 3 beta (GSK3β) which leads to stabilisation of CRY2 and faster degradation of REV-ERBα protein levels [131].

Further compounds have been described as targeting the **core clock genes**; most prominently, direct or indirect modulators of *casein kinase 1* [132, 133] but also *RevErbα* [134] and retinoic acid receptor-related orphan receptor *α*/γ (ROR*α*/γ) [135] protein products. In recent years, multiple high throughput forward screening (HTS*) in vitro* projects have been undertaken to find novel chronomodulatory small molecules. So far, modulators for three targets have been reported: *RevErbα*, *Cryptochrome*, and *Casein kinase 1* [32, 136-139]. For these experiments, circadian real-time reporter expressing cell lines, mostly the human osteosarcoma cell line U2OS have been used. Among these agents, the CRY modulators have already been translated into clinical Proof-of-Concept trials in two indications, Cushing syndrome and Diabetes mellitus type 2. It remains to be seen, which of the discovered mechanisms of action will effectively prove to be useful in a clinical setting and whether clock alterations do not lead to unsuspected adverse events.

Concluding Remarks

Circadian clocks modulate many molecular pathways of human physiology and pathophysiology. An increasing amount of evidence indicates that there is a biologically and medically relevant impact of time-of-day on pharmacotherapy. Recent chronopharmacology studies involving cancer, rheumatolology, hematology, neurologic /psychiatric disorders and cardiovascular medicine have been undertaken (Tables 1&2). Indeed, circadian clocks modulate many processes that define drug properties and behaviour. There have been a few successes in the clinical translation of

chronotherapy, but nevertheless, the medical community, drug developers and, importantly, regulatory agencies, have yet to embrace circadian timing as an important factor modulating both the efficacy and safety of pharmacotherapy. The identification of reliable and cost-effective biomarkers of the CTS might indeed represent the major effort that is required to fulfil the already documented promise of chronotherapy for improving outcomes of patients with various diseases. As such, this is an exciting era to be integrated into the development of both new drugs, and new clock-based therapeutic strategies. Our ability to pharmacologically target the CTS to alleviate or treat certain chronic diseases will bring the next step in fully implementing the concept of chronomedicine (see Outstanding Questions).

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Figure Legends

Figure 1: The cell-autonomous circadian clockwork (left) is the functional unit of the circadian timing system, and determines the complex interaction with xenobiotic metabolism.

(Left) Simplified core circadian oscillator (See Box 1 for details and abbreviations) and one output relevant for xenobiotic metabolism through control of aminolevulinic acid synthase 1 (ALAS1), constitutive androstane receptor (CAR) and cytochrome P450 oxidoreductase (POR).

(Right) The CTS involves a central hypothalamic pacemaker – the suprachiasmatic nuclei (SCN) – which coordinates clocks in all the cells in the body through the generation of an array of physiological rhythms such as rest-activity, body temperature and hormonal secretions. The SCN synchronises the peripheral clocks relative to each other and to the environmental time cues provided by the day-night and social cycles (blue box). Following exposure of an organism to a xenobiotic, the substance undergoes the classical Absorption, Distribution, Metabolism and Elimination (ADME) processes. All of these processes, which will ultimately determine the toxicity or pharmacologic effect of the xenobiotic are regulated by peripheral and central clocks present in the gut, heart and blood vessels, liver and pancreas, as well as kidney and colon. Xenobiotics can also reset the molecular clock or CTS through direct interference with the molecular clock or by altering or disrupting physiological pathways.

Tables

Table 1. Recent examples of medications with chronopharmacologic effects and corresponding clock-controlled metabolic pathways. Abbreviations: dosing routes p.o., oral; i.v. intravenous; i.t., intratracheal; i.p., intraperitoneal; LLC: lewis lung carcinoma; Ref reference; ZT zeitgeber time; C_{max} peak plasma concentration; >> higher/better/more than.

Agent (Target)	Circadian modulation	Dosing, species	Main findings	Ref
Erlotinib (Tyrosine kinase inhibitor, anticancer drug)	EGFR, *Ras*/*Raf*/MAPK & PIK3/AKT (tumour)	5 mg/kg/d p.o., subchronic Mouse (female) with Hec827 xenograft	Tumour inhibition ZT1 >> ZT13	[140]
	Cyp3a11, *Cyp3a13*, *Cyp1a2* (liver)	3 mg p.o., single dose Mouse (female) with LLC xenograft	Systemic exposure ZT1>>ZT13	[141]
Sunitinib (Tyrosine kinase inhibitor, anticancer drug)	*Cyp3a11* (Liver, duodenum, jejunum) *abcb1a* (Liver, duodenum, jejunum, lung)	1.06 mg p.o., single dose Mouse (female)	Systemic and liver exposure to sunitinib ZT20>ZT0 SUI112 (metabolite) ZT8>ZT12	[142]
	(elimination)*	25 mg p.o., single dose Rabbit (male)	C_{max} and systemic exposure to sunitinib and SUI12662 (metabolite): ZT1>>ZT13 Clearance faster after ZT13 dosing	[143]
Lapatinib (dual tyrosine kinase inhibitor interrupting the HER2/neu and EGFR pathways, anticancer drug)	EGFR/*Ras*/*Raf*/MAPK *Errfi1*, *Dusp1* (liver) *Hbegf*, *Tgfα*, *Eref* (liver)	40 mg/kg/d, p.o., subchronic Mouse (male) EGFR/HER2 driven tumour	Tumour and angiogenesis inhibition ZT23>>ZT13	[19-22]

Roscovitine (seliciclib, CDK inhibitor, anticancer drug)	*Cyp3a11*, *Cyp3a13* (liver)	300 mg/kg/d p.o., single Mouse (male)	Systemic, kidney, adipose tissue exposure ZT3>>ZT19 Liver metabolic ratio ZT19>ZT3	[144]
Everolimus (mTOR inhibitor, anticancer drug, immunosuppressant)	mTOR/*Fbxw7*/P70S6K (tumour)	20 mg/kg i.v., single Mouse (male) w/o renal cell tumour	Plasma PK ZT12 = ZT0 Antitumour efficacy ZT12 >>ZT0	[145]
Irinotecan (Top1 inhibitor, anticancer drug)	*Ces2*, *Ugt1a1*, *abcb1a*, *abcb1b* (liver and ileum), *abcc2* (ileum)	50-80 mg/kg i.v., single or 4d repeat dosing Mouse (male & female, 4 strains)	Least toxic time and chronoPK-PD relation dependent on sex and strain ZT7, ZT11 or ZT15	[20]
Tamoxifen (antiestrogenic,, anticancer drug)	*Cyp2d10*, *Cyp2d22*, *Cyp3a11* (liver)	4 mg p.o., single Mouse (female)	Plasma and liver exposure ZT18>=ZT6 (trend)	[146]
Pethidine (analgesic opioid)	N-demethylation*	20 mg/kg/d i.p., single or 5 d Mouse (male)	Analgesic effect & metabolism ZT15 > ZT3	[147]
Bleomycin (toxicant and anticancer drug)	NRF2/glutathione antioxidant defense	1 mg/kg i.t., single Mouse (female)	Pulmonary fibrosis ZT12>>ZT0	[148]
Tolbutamide (antidiabetic)	Glucose transporter 4 (GLUT4)	5-10 mg/kg i.v., single Rat (male)	Hypoglycaemia ZT12>>ZT0	[149]
Isoniazid (antituberculous antibiotic)	(N-acetyltransferase 2-NAT2 and *Cyp2e1*)*	120-180 mg/kg i.p., single Mouse (male)	Gross and hematologic toxicities ZT1<ZT9	[150]
Acetaminophen (analgesic)	*Cyp2e1*, *Por* (liver)	250 mg/kg, i.p. single Mouse (male)	Toxicity ZT2<<ZT14	[58, 59, 151]
Pentobarbital (hypnotic, , antiepileptic)	*Por* (liver)	50-60 mg/kg, i.p. single Mouse (male)	Sleep time ZT2>>ZT14 Clearance ZT2<ZT14	[59]

*Pathways or enzymes indicate suggested mechanisms for effects

519 **Table 2.** Recent clinical chronotherapy studies. Abbreviations: pts, patients; 5-FU-LV, 5-fluorouracil-leucovorin; I-OHP,
520 oxaliplatin; PK pharmacokinetics; N, number of subjects; M, male; F, female; AUC, Area under the curve; C_{max}, peak plasma
521 concentration; gr, grade; $C_{through,}$ Minimum drug concentration in blood/plasma in multiple (subsequent) dosing at steady-state condition.

Disease	Drug(s) (dose, route)	Study design	Dosing schedule	N	Main findings	Ref
Breast cancer (hormone receptor receptive)	Tamoxifen (20 or 40 mg p.o.)	PK cross over	8:00 vs 13:00 vs 20:00 (4 weeks on each dosing time)	27 F	Mean C_{max} and AUC_{0-8h} of tamoxifen and endoxifen (bioactive metabolite) 8:00 >> 20:00 (by ≈ 20%) Mean t_{max} 8:00 < 20:00 High CYP2D6 metabolism may enhance circadian effect	[146]
Renal cell cancer, gastrointestinal stromal, or pancreatic neuro-endocrine tumours	Sunitinib (stable once daily dose for > 2 weeks before entry)	PK randomized cross over	8:00 vs 18:00 (3 weeks on each dosing time) Additional testing of 13:00 for pts subset	27 pts (22 M, 5 F) 12 pts: 3 dosing times	Mean concentration at time of subsequent dose intake ($C_{trough)}$) : (13:00 = 18:00) > 8:00 No difference in AUC	[142]
Non small cell lung cancer (advanced)	Cisplatin (20mg/m²/d x 4d, combined with docetaxel or gemcitabine)	Randomized Phase II with minimization	6:00 vs 18:00	41 pts (28 M, 13 F)	Neutropenia gr 3-4: 12% at 18:00 vs 33% at 6:00 Nausea gr 1-2: 18:00 < 6:00 Total and unbound cisplatin clearance 18:00 > 6:00	[152]
Metastatic colorectal cancer	5-FU-LV and l-OHP (5-FU 3000-3600 mg/m², LV 1200 mg/m², l-OHP 100 mg/m², q 2 wks	International randomized Phase III (post hoc analysis)	Fixed chronomodulated delivery (chronoFLO4) vs conventional delivery (FOLFOX2)	556 pts (331 M, 225 F)	Neutropenia - All grades: chronoFLO4, 33%, FOLFOX, 61% - Grade 3-4: chronoFLO4, 7% FOLFOX, 25% - More frequent in women - Predictive of a better survival for FOLFOX2, not chronoFLO4	[153]

Metastatic colorectal cancer	5-FU-LV and l-OHP (5-FU 3000-3600 mg/m², LV 1200 mg/m², l-OHP 100 mg/m², q 2 wks	International randomized Phase III (post hoc analysis)	Fixed chronomodulated delivery (chronoFLO4) vs conventional delivery (FOLFOX2)	556 pts (331 M, 225 F)	Neutropenia - All grades: chronoFLO4, 33%, FOLFOX, 61% - Grade 3-4: chronoFLO4, 7% FOLFOX, 25% - More frequent in women - Predictive of a better survival for FOLFOX2, not chronoFLO4	[153]
	5-FU-LV and l-OHP (5-FU 3000-3600mg/m², LV 1200-1500mg/m², l-OHP, 100-125mg/m², q 2-3wks)	Meta-analysis of 3 international Phase III randomized	ChronoFLO vs Conv (FOLFOX2 or constant rate infusion)	842 pts (497 M, 345 F)	Sex-dependent efficacy of optimal fixed schedule : - Median survival Male: ChronoFLO: 20.8 mo Conv : 17.5 mo - Median survival Female: ChronoFLO: 16.6 mo Conv : 18.4 mo - Same sex- schedule interaction for progression-free survival and tumour response rate in pooled analysis and for each randomized trial.	[154]
Rheumatoid arthritis (RA)	Low dose modified release prednisone (5 mg, MR prednisone)	12-week double-blind placebo-controlled randomized (CAPRA2)	Evening intake vs placebo combined with existing RA disease – modifying antirheumatic drug (DMARD) treatment	350 pts	- 20% improvement in RA signs and symptoms: MR prednisone: 48% vs Placebo: 29% - 50% improvement: MR prednisone: 22% vs Placebo: 10%	[155]
					MR prednisone vs placebo: - reduced fatigue - improved SF 36 vitality score and other well-being parameters	[156]
Adrenal congenital hypoplasia	Chronocort (10 mg at 7:00 and 20 mg at 23:00)	PK Phase II	Unequal dosing morning and evening	16 pts (8 M, 8 F)	- Good approximation of circadian physiologic secretion - Good tolerability and effectiveness in controlling androgen excess	[157]

Chronic kidney disease	Valsartan (80-320 mg p.o.)	Randomized	Bedtime vs awakening	60 pts (non dipper) 30 pts (dipper)	Non dippers on bedtime vs awakening Valsartan: - greater reduction in proteinuria - better glomerular filtration rate - better protection against myocardial hypertrophy	[158]
	Blood pressure lowering agent	Systematic review of 7 trials	Bedtime vs no bedtime	1277 pts	BP lowering medication at bedtime reduced total events and major cardiovascular events Non significant reduction of death rate ($p \approx 0.06$)	[159]
Atherothrombosis (post myocardial infarction)	Clopidogrel (75 mg p.o.) and aspirin (75 mg p.o.)	Randomized	6:00 vs 10:00 vs 14:00 vs 19:00 for 4 days on each dosing time	59 pts (45 M, 14 F)	Platelet inhibition lowest after dosing at 10:00 Non responsiveness: 2.4-fold more frequent at 10:00 vs 6:00	[160]
Osteoporosis (postmenopausal)	Raloxifene (estrogen receptor modulator, 60 mg p.o.)	Randomized	Morning vs evening for 12 months	39 healthy (F)	Plasminogen activation inhibitor1: Morning dosing: +40% Evening dosing: -0.3% In favour of increased risk of venous thromboembolism after morning dosing	[161]
Endogenous coagulation	Rivaroxaban (anticoagulant agent, 10 mg p.o.)	Randomized controlled cross over	Morning vs evening for 3 days	16 healthy	Plasma concentration 12 h after dosing: Evening : 53.3 ng/mL Morning : 23.3 ng/mL Evening dosing: better matching physiologic morning hypo-fibrinolysis	[162]

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Text Boxes

Text Box 1: The Molecular clockwork

The unit of the molecular circadian oscillator is the cell. At the core of this cell-autonomous molecular mechanism driving circadian cycles are two interlocked transcriptional/translational feedback loops. The mechanistic principle of a circadian clock is rather simple: an activator gene initiates transcription of a repressor gene. Then, the repressor protein re-enters the nucleus and eventually shuts off its own transcription until the repressor is degraded and the cycle can start again [163]. In mammals, *Bmal1* is the key transcriptional activator. BMAL1 binds to regulatory E-box elements as a complex with its dimerization partners CLOCK or NPAS2 [164] and activates the transcription of *Period* (*Per*) and *Cry* (*Cryptochrome*) genes. After translation, PER and CRY proteins re-enter the nucleus and as part of a large complex repress their own transcription [165]. Once the repressor complex dissociates, the cycle can start once more. A second loop stabilizes this basic loop: as in the case of *Per*s and *Cry*s, *RevErb*s and ROR orphan nuclear receptor family genes are activated by the BMAL1 containing complex binding to the E-box on their promoters. In turn, ROR and REVERB proteins competitively bind to ROR-elements, activating and repressing *Bmal1* transcription, respectively [166]. Most important for the usefulness of any clock are its hands, i.e., the output. In mammals, about 20-40% of the transcriptome [106], proteome [104, 105] and metabolome [114, 116] are modulated by the circadian clock. Importantly, many rate-limiting steps of key physiological pathways including those important for drug pharmacokinetics and pharmodynamics are under direct or indirect clock control [10, 106, 167]. Post-transcriptional modifications of RNA [168], the regulation of ribosomal translation [169, 170] as well as post-translational control by kinases, phosphatases and acetylases have been implicated in the daily variation and tuning of the circadian clock [171, 172]. Possibly completely independent of the transcriptional feedback loop, non-transcriptional oscillators have been described; for example, the peroxiredoxin oscillations in human red blood cells [173].

Glossary Box

- **Circadian Timing System (CTS)**, In mammals, the circadian timing system consists of three levels of interacting mechanisms: (i) *the cell-autonomous molecular circadian clock*, (ii) the suprachiasmatic nuclei (SCN), and (iii) *physiological rhythms*.
- **Circadian rhythm,** A temperature compensated biological rhythm with a period of about one day (lat. circa, about; dies, day), which persists in constant conditions without any time cues, i.e., is endogenous.
- **Core clock genes**, are an integral part of the core clock mechanism and most of them physically interact with one another (Box 2). In mammals they are to some degree redundant (e.g., *Pers*, *Crys*). Knock-out studies in mice suggest that all of them are important for proper clock function, at least in some tissues. In comparison, clock-controlled genes, are genes with significant circadian modulation in their expression profile that do not feedback on the clock mechanism themselves. Typically, these genes are driven by promoter elements like E- or D-box elements, but might also contain further tissue-specific regulatory elements that lead to tissue-specific inducibility.
- **Physiological rhythms**, provide the endogenous time cues needed for the daily coordination and resetting of cellular clocks in the peripheral tissues of an organism.
- **Chronotype**, or the diurnal preference of an individual is based at least partially genetically determined, but is plastic to a certain degree. Previously, variation of chronotype with age, sex and behaviour (e.g., shift-work, habits) have been described.
- **Xenobiotic**, a chemical compound such as a drug, a toxicant, a pesticide, or a carcinogen that is foreign to a living organism.
- **Suprachiasmatic nuclei (SCN)** or **central clock,** this paired structure in the ventral hypothalamus is indispensible for generating most consolidated circadian physiological and behavioural rhythms. They are considered the central pacemaker and receive light input from the retina and synchronise the organism with environmental day/night cycles. In contrast, **peripheral clocks** are all non-SCN tissues or organs.
- **Non-photic signals**, cues other than the alternation of light and darkness. Food, activity and few other so-called zeitgeber have been shown to be able to influence the circadian system and if rhythmically presented synchronise the CTS.
- **Circadian amplitude and phase** are two parameters which characterise the extent of variation and the timing of a rhythm with an about 24-hour period.

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Trends Box

- The Circadian Timing System (CTS) significantly modulates efficacy and toxicity of many xenobiotics and therefore, time-of-day is an important variable to consider for many marketed drugs, as well as drugs under development, and environmental toxicant exposure.

- Cell-autonomous circadian oscillations in peripheral tissues have been shown to play essential roles in time-of-day variations, and might present novel targets for pharmacotherapy.
- Lifestyle, sex, age, genotype, disease, and xenobiotic effects can shape and alter CTS dynamics, including clock-controlled metabolism pathways.
- Recent small molecule drug screens have identified several compounds that target the circadian clockwork itself, and might be useful to treat circadian desynchronisation due to disease or other drug or toxicant effects.

Outstanding Questions Box

- The pharmaceutical industry should consider the integration of chronopharmacology into new drug development as a competitive advantage for safer and more effective medications. Similarly, regulatory agencies should request circadian timing studies to complement dose-effect and safety studies of pharmacological agents. Which scientific and biomedical framework will prompt this to happen?
- How will Circadian Timing System status and clock phase be reliably assessed using minimally invasive single sampling procedures in a given tissue in human patients, in order to predict optimal treatment timing?
- Will *in vitro* chronopharmacology/chronotoxicology provide a robust tool for the identification of xenobiotic timing with best tolerability and/or optimal efficacy?
- Will a comprehensive systems medicine approach help integrate potential CTS-modifiers, including disease, lifestyle, aging, sex and genetics to achieve optimal personalized drug dosage and timing?