Circadian Timing in Cancer Treatments

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Annu. Rev. Pharmacol. Toxicol. 2010. 50:377–421

First published online as a Review in Advance on November 17, 2009

The *Annual Review of Pharmacology and Toxicology* is online at pharmtox.annualreviews.org

This article's doi: 10.1146/annurev.pharmtox.48.113006.094626

0362-1642/10/0210-0377$20.00

Key Words

molecular clock, drug delivery, chronotherapeutics, gender, mathematical models, clinical trial methodology, systems biology, personalized medicine

Abstract

The circadian timing system is composed of molecular clocks, which drive 24-h changes in xenobiotic metabolism and detoxification, cell cycle events, DNA repair, apoptosis, and angiogenesis. The cellular circadian clocks are coordinated by endogenous physiological rhythms, so that they tick in synchrony in the host tissues that can be damaged by anticancer agents. As a result, circadian timing can modify 2- to 10-fold the tolerability of anticancer medications in experimental models and in cancer patients. Improved efficacy is also seen when drugs are given near their respective times of best tolerability, due to (*a*) inherently poor circadian entrainment of tumors and (*b*) persistent circadian entrainment of healthy tissues. Conversely, host clocks are disrupted whenever anticancer drugs are administered at their most toxic time. On the other hand, circadian disruption accelerates experimental and clinical cancer processes. Gender, circadian physiology, clock genes, and cell cycle critically affect outcome on cancer chronotherapeutics. Mathematical and systems biology approaches currently develop and integrate theoretical, experimental, and technological tools in order to further optimize and personalize the circadian administration of cancer treatments.

INTRODUCTION

UGT: UDP-glucuronosyl transferase

5-FU: 5-fluorouracil

Circadian: biological rhythm with an about one day period (circa, about; dies, day)

Chronotherapeutics: the administration of treatments according to circadian or other biological rhythms

Biological rhythm: self-sustained and endogenous biological oscillation

Period: cycle duration

Circadian timing system (CTS): the biological system that generates ~24 hour rhythms in cellular and organism physiology and adjusts them to environmental cycles

The outcomes of patients receiving anticancer treatments remain complicated by unpredictable severe toxicities and/or poor antitumor efficacy (1). Greater than 10-fold interindividual changes in drug exposure and pharmacokinetic parameters as well as the variable status of gene expression and metabolism within the tumor itself likely contribute to large interpatient variability in therapeutic index (1, 2). Whereas mapping the genetic polymorphisms in drug metabolism and detoxification can predict undue drug toxicity, the identification of molecular signatures in tumor cells can predict efficacy of specific anticancer drugs (3, 4). Recent results, however, emphasize that the relevance of the UDP-glucuronosyl-transferase *Ugt1a1*28* polymorphism, an FDA-approved test for the prediction of irinotecan toxicity, varies according to gender, delivery schedule, drug dose level, and associated genetic polymorphisms (5). Similar findings are reported for the polymorphisms of *Dpyd*, which encodes for dihydropyrimidine dehydrogenase, the rate-limiting enzyme for the catabolism of 5-fluorouracil (5-FU) (6).

The large variability in the outcome of patients on anticancer therapy is paralleled by the limited success rate of anticancer drug development. Only 5% of the anticancer agents selected for clinical development successfully complete all clinical phases and become registered as medications. Poor prediction of safety is identified as the main cause for interrupted clinical development (7).

This review emphasizes that treatment timing within the 24-h timescale, that is, circadian (circa, about; dies, day) timing, can predictably change by severalfold the tolerability and the antitumor efficacy of anticancer agents both in experimental models and in cancer patients.

Indeed, most biological functions display circadian changes in mammals (8). The disruption of circadian clocks that drive these rhythms favors cancer processes and reduces survival in cancerous rodents and human patients (9–14). A recent monograph by the International Agency for Research on Cancer (World Health Organization) concludes that "shift work that involves circadian disruption is probably carcinogenic to humans" with an estimated risk level 2A, that is, close to full evidence (15). Thus, the prevention of circadian disruption, and/or the restoration of functional clocks, could constitute new objectives for therapeutics.

Chronotherapeutics aims at improving the tolerability and/or the efficacy of medications through the administration of treatments according to biological rhythms (8, 16). The adequate adjustment of treatment delivery to physiological rhythms and the restoration or the induction of these rhythms can improve therapeutic outcomes in cancer patients (8, 17).

Recent advances identify critical molecular events that rhythmically control drug metabolism and detoxification, cell cycle, molecular targets, DNA repair, apoptosis, and angiogenesis. The coordination of these processes along the 24-h period is ensured by the circadian timing system (CTS), whose hierarchical organization determines chronotherapeutic effects. Phase I to III clinical trials validate the relevance of circadian timing of cancer treatments. Moreover, translational studies identify potential key determinants to optimally shape circadian drug delivery patterns in a given patient. Data-based computational models are providing novel insights into the interactions between circadian clocks, cell cycle, and anticancer drug pharmacology. They now reveal several critical dynamic events for the success of cancer chronotherapeutics through the design of patient-tailored chronomodulated delivery of anticancer medications.

THE CIRCADIAN TIMING SYSTEM

Overall Organization

The CTS coordinates physiology and cellular functions over a 24-h period. Environmental synchronizers such as the alternation of days and nights, socio-professional routines, and meal times

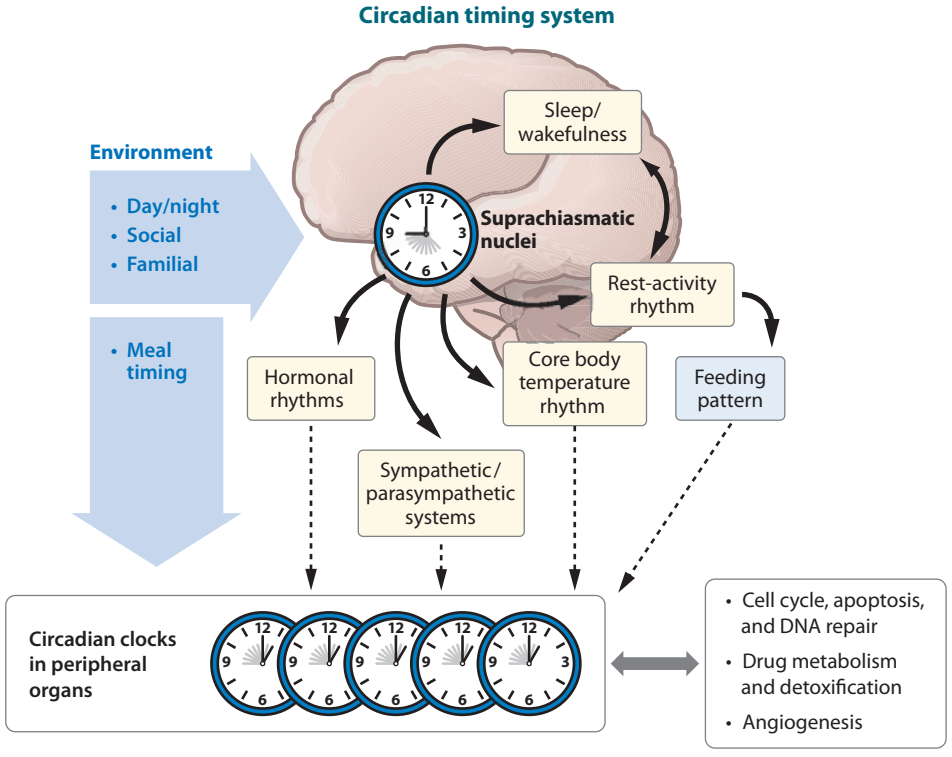


Figure 1

Schematic representation of the CTS. The CTS is composed of (*a*) a hypothalamic pacemaker, the suprachiasmatic nuclei SCN, (*b*) an array of SCN-generated circadian physiology outputs, and (*c*) molecular clocks in the cells of all peripheral tissues. Molecular clocks rhythmically control xenobiotic metabolism and detoxification, cell cycle, apoptosis, DNA repair, and angiogenesis over a 24-h period. The CTS is synchronized with time cues provided by light-dark cycles and other environmental factors. Circadian physiology outputs can also serve as CTS biomarkers.

entrain and calibrate at precisely 24 h, the period of the CTS (**Figure 1**). Endogenous circadian rhythms with periods differing from precisely 24 h characterize all aspects of mammalian physiology (10, 18, 19). In human beings synchronized with usual light-dark, socio-professional, and feeding synchronizers, motor activity is high at daytime and low at night, body temperature reaches a maximum in the early evening, cortisol secretion by the adrenal gland rapidly rises from a nadir near 2:00 a.m. to a maximum near 8:00 a.m., and melatonin secretion by the pineal gland mostly occurs at night, with a maximum near 2:00 a.m. (18, 19). This circadian physiology is generated or controlled by a central pacemaker, the suprachiasmatic nuclei (SCN), in the hypothalamus. The circadian period of the SCN neurons is calibrated to 24 h through the perception of synchronization signals, namely light and darkness via the retino-hypothalamic tract using glutamate and pituitary-adenylate-cyclase-activating peptide (PACAP) as neuromediators and other brain areas via neuropeptide Y fibers (18). The SCN generates circadian physiology through diffusible signals, including transforming growth factor α, epidermal growth factor, prokineticin-2 (PK-2), cardiotrophin-like cytokine, and neuroanatomic sympathetic and parasympathetic pathways (20–22). Circadian physiology and other signals directly or indirectly emanating from the SCN coordinate molecular clocks in each cell (18, 23). In turn, the molecular clock rhythmically

controls many cellular functions that are relevant for cancer treatment including drug metabolism and detoxification as well as cellular proliferation, DNA damage sensing and repair, apoptosis, and angiogenesis (24).

The periodic resetting of the circadian time structure by external 24-h cycles allows for the prediction of times of the peaks and troughs of circadian rhythms in rodents and in humans. This applies to the rhythms that regulate anticancer drug pharmacology and cellular proliferation (23, 24). Conversely, a lack of external synchronizers, that is, a defect in the perception of environmental time cues through blindness, for instance, or an alteration of the circadian physiology, molecular clock, or clock-controlled pathways, results in the deregulation of the circadian time structure (19, 25, 26). In turn, relevant 24-h rhythms become damped, ablated, or phase shifted, with an unpredictable timing of the peaks and troughs if the circadian period is lengthened, shortened, or shifted. In such cases, melatonin, glucocorticoids, or other chronobiotic agents can restore proper circadian coordination (26, 27).

Healthy human subjects can display different CTS phasing, despite exposure to the same environmental synchronizers. Such distinct chronotypes are defined with questionnaires on living habits, which reflect distinct timing of circadian behavior, physiology, and clock gene expression patterns (28).

Clock: circadian locomotor output cycles kaput

Bmal: brain and muscle aryl hydrocarbon receptor nuclear translocator

Per: period (gene or protein)

Cry: cryptochrome

DBP: albumin D-binding protein

TEF: thyrotroph embryonic factor

HLF: hepatic leukemia factor

Circadian Clock Mechanisms

A dozen specific clock genes constitute the core of the molecular clock in mammals (**Figure 2**). These genes are involved in transcriptional and posttranscriptional activation and inhibition regulatory loops that result in the generation of the circadian oscillation in individual mammalian cells. In particular, the CLOCK-BMAL1 or NPAS2-BMAL1 protein dimers play a key role in the molecular clock through the activation of the transcription of the clock genes *Per* and *Cry* (23, 27). The functionality of the molecular clock in peripheral tissues including malignant tumors can be estimated through the relative phase relations of circadian expression patterns of three core clock genes whose transcription is regulated by one another: *Rev-erbα* downregulates *Bmal1*, *Bmal1* upregulates *Rev-erbα* and *Per2*, and *Per2* downregulates *Rev-erbα* and its own transcription (23, 24).

The CLOCK-BMAL1 transactivation complex also rhythmically controls the mRNA transcription of proline-acidic amino acid–rich basic leucine zipper (PAR bZip) transcription factors, including albumin D-binding protein (DBP), thyrotroph embryonic factor (TEF), and hepatic leukemia factor (HLF) (29). These transcription factors regulate most pathways that handle xenobiotic metabolism and detoxification in liver, intestine, and kidney through the rhythmic control of C-androstane receptor, P450 oxydo-reductases, and 5-amino-δ-levulinic acid synthetase (*Alas1*) (23, 29). Furthermore, posttranslational modifications regulate ticking of the molecular clock (30).

The CLOCK-BMAL1 dimer also gates cell cycle phase transitions through the repression of *c-Myc* and *p21*, two important players in cellular proliferation and apoptosis, the activation of *p53*, a proapoptotic gene, and that of *Wee1*, whose protein gates transition from G_2 to mitosis (24, 31, 32). Circadian clocks further regulate apoptosis through the rhythmic expressions of antiapoptotic BCL-2 protein and proapoptotic BAX protein (33); DNA damage sensing through molecular interactions of ataxia telangiectasia mutated (ATM) or ATM and rad3-related interacting protein (ATRIP) with clock proteins PERs, CRYs, and TIM (32, 34); and DNA repair through rhythmic activities or levels of O_6-methylguanine DNA methyltransferase, a protein that excises lethal DNA alkylated lesions produced by nitrosoureas (35), as well as *Tip60*, *Xpa*, and possibly *Ercc1*, which repair platinum-induced DNA adducts (36–38).

The intrinsic sustainability of molecular clocks has been shown in synchronized cell cultures. Thus, cell lines are potential models for in vitro studies of circadian clocks and clock-controlled

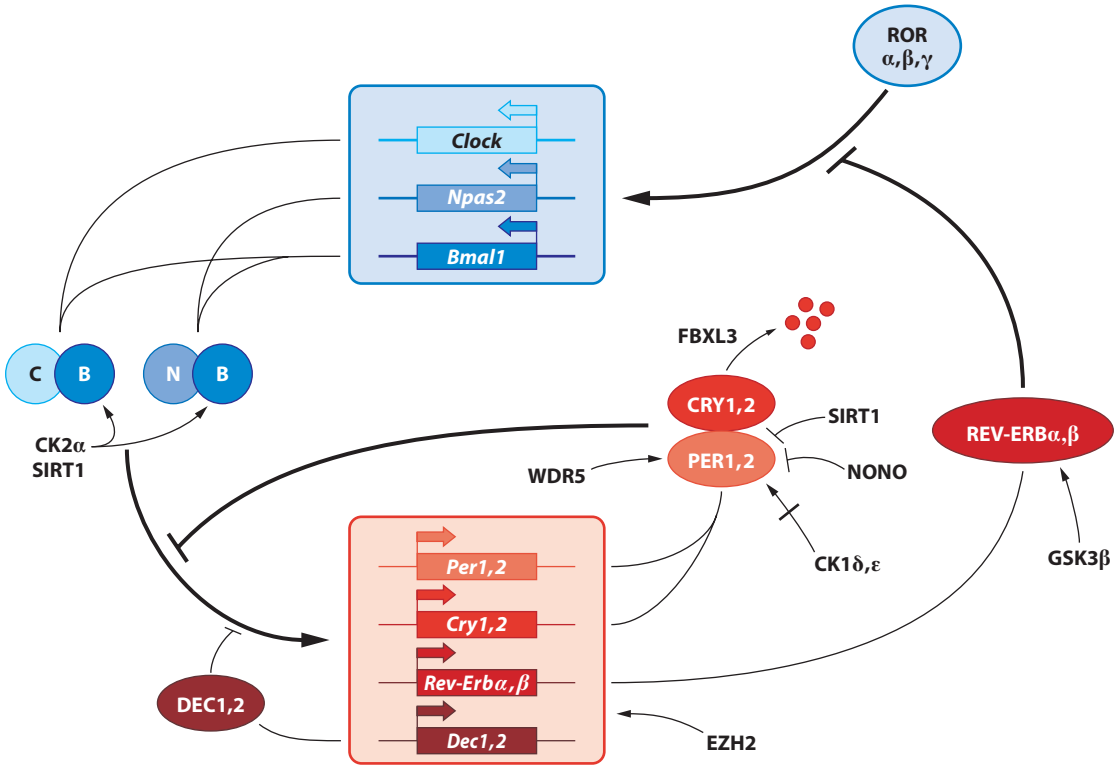


Figure 2

Simplified hypothetical mammalian circadian clock. The molecular oscillator is thought to be based on molecular feedback loops within a positive limb (CLOCK, NPAS2, BMAL1) and a negative limb (PER and CRY) that are interconnected via the nuclear orphan receptor REV-ERBα. The transcription of *Per* and *Cry* genes is activated by heterodimers between BMAL1 (B) and either of the two related proteins CLOCK (C) or NPAS2 (N). The polycomb protein EZH2 as well as casein kinase 2 (CK2) and silencing information regulator SIRT1 interact with these heterodimers and thereby facilitate their action. The accumulation and activity of PER and CRY proteins are also influenced by phosphorylation by protein kinases (CK1δ,ε), by ubiquitination via a complex containing the F-box protein FBXL3 (specific for CRYs), by the histone methyl-transferase-binding protein WDR5, and by NONO, an RNA- and DNA-binding protein. DEC1 and DEC2 compete with BMAL1-CLOCK/NPAS2 heterodimers for E-box binding and thereby reduce E-box-mediated transactivation. An accessory feedback loop, employing the nuclear orphan receptors RORα, RORβ, and RORγ as activators, and REV-ERBα and REV-ERBβ as repressors, regulates the circadian transcription of *Bmal1*. (Adapted from U. Schibler, with permission.)

pathways (39). Two-hour exposure of cultured cells to 50% horse serum, dexamethasone, or other compounds synchronizes the circadian clocks in cultured cells whose internal timing is otherwise drifting at a different pace (39, 40). Circadian transcription has been demonstrated for at least three full periods in synchronized cultures of cell lines and ex vivo cellular preparation or tissue explants from rodents or humans, including SCN, liver, lung, kidney, intestine, and adipose tissue (41–43). The use of a PERIOD2-LUCIFERASE fusion protein as a real-time reporter of circadian dynamics demonstrates that peripheral tissues from mice self-sustain circadian oscillations for >20 cycles in isolation, with tissue-specific differences in circadian period and phase (44). Repeat serum shocks at 3-day intervals or 24-h cycles in external temperature avoid the desynchronization of in vitro transcription circadian rhythms (39, 45). The properties of synchronized cell cultures thus support their recent use as potential models for cellular chronopharmacology.

THE EXPERIMENTAL CHRONOPHARMACOLOGY OF ANTICANCER AGENTS

ZT: Zeitgeber time (equivalent to hours after light onset)

The Relevance of Circadian Timing for Treatment Tolerability

Circadian timing largely modifies the extent of toxicity of 40 anticancer drugs, including cytostatics, cytokines, and targeted biological agents, in mice or rats (**Table 1**). A potentially lethal dose of any of these agents results in 2-fold to more than 10-fold changes in the incidence of toxic deaths and/or maximum body weight loss as a function of circadian timing of drug administration. Such large differences occur irrespective of delivery route—oral, intravenous, intraperitoneal, or intra-arterial—or the number of daily or weekly administrations (23). The methodology used to demonstrate the 24-h changes in anticancer drug tolerability involves the synchronization of nocturnally active mice or rats with an alternation of 12 h of light and 12 h of darkness (LD12:12). The same drug dose is administered to different groups of rats or mice, with each group corresponding to a different circadian stage, also called Zeitgeber time (ZT). Usually, six circadian stages, occurring 4 h apart, are tested. Time usually is expressed in ZT hours or in hours after light onset. Dedicated chronobiologic animal facilities allow setup light onset at the desired time for different groups of animals located on different isolated shelves, so that different circadian stages are tested at the most convenient times (46). **Figure 3** depicts the times of least toxicity and benefit from optimal circadian timing, referring to external LD synchronizer and internal average body temperature rhythm for 16 anticancer drugs in male B6D2F1 mice (female C57BL/6 × male DBA2) in studies performed at our laboratory (47–63). The optimal circadian timings are staggered along the 24-h period and cannot be predicted thus far by the knowledge of pharmacologic class or that of main target organs for toxicity. Circadian rhythms in the tolerability of anticancer drugs persist in rodents kept in constant darkness or in constant light, which demonstrates their endogenicity (64).

Even when combined, chemotherapeutic agents display the least toxicity near their respective times of best tolerability as single agents, as shown for doxorubicin-cisplatin in Lou rats, irinotecan-oxaliplatin or gemcitabine-cisplatin in B6D2F1 mice, and docetaxel-doxorubicin in C3H/He mice (56, 65–67). These findings support the persistence of the circadian control of anticancer drug determinants after exposure to the first anticancer agent, at least when the latter is given near the time of best tolerability.

Circadian Control of Metabolism, Detoxification, and Pharmacokinetics

Most anticancer agents with circadian tolerability undergo oxidation, reduction, or hydrolysis under phase I metabolism, mainly in the liver and, to a lesser extent, in the intestine (1, 2). The CTS controls both phase I metabolism and phase II drug detoxification and elimination through redundant processes involving rhythmic physiology and circadian clock signaling (**Figure 4**) (23, 29, 68, 69).

The activity of most microsomal oxidases are highest by severalfold during the dark (activity) span and lowest during the light (rest) span in the liver of rats and mice (29, 68, 70). Twenty-four-hour rhythms further characterize the activities of several CYP isoenzymes (23, 71). Two- to eight-fold circadian changes in mRNA expression are found for cytochrome P-450 oxidoreductase in liver and intestine, with a maximum at ZT12; for *Cyp2b10* (testosterone 16-α-hydroxylase), with a maximum at ZT16 in liver and intestine; and for *Cyp2c50*, with a maximum at ZT20 in mouse liver (29). However, *Cyp3a13* may escape from clock regulation (72).

The circadian rhythm in *Cyp3a* likely contributes to the chronotolerance pattern of seliciclib, docetaxel, irinotecan, mitoxantrone, and vinorelbine, which undergo oxidative metabolism. On

Table 1 Anticancer drugs with documented relevance of circadian timing for tolerability, pharmacokinetics, and/or antitumor efficacy in laboratory rodents

Pharmacologic class	Drug	Endpoint modified by circadian timing [reference(s)]		
		Tolerance	Pharmacokinetics	Efficacy
Antimetabolite	D-actinomycin	(219)		
	Methotrexate	(220, 221)	(99, 221)	(99)
	5-fluorouracil	(88, 222, 223)	(86, 224)	(88, 225)
	Floxuridine	(226, 227)		(226, 228)
	Arabinofuranosylcytosine	(229)		(229, 230)
	Gemcitabine	(56)		(56)
	L-alanosine	(55)		(55)
Top 1 inhibitor	Irinotecan	(48, 77, 82)	(77, 82)	(66)
	Topotecan	(95)		(95)
	9-aminocamptothecin	(231)		(231)
Top 2 inhibitor	Mitoxantrone	(54)	(54)	
	Etoposide	(52)		
DNA intercalator	Daunorubicin	(232)		
	Doxorubicin	(64, 67, 233, 234)	(234)	(65, 67, 235, 236)
	Doxorubicin-liposomes	(237)		(237)
	Theprubicin	(46, 53, 238)		(239)
	Epirubicin	(240)		
Mitotic inhibitor	Vincristine	(241)		
	Vinblastine	(242)		
	Vinorelbine	(63, 182)		(182)
	Docetaxel	(58, 67)		(58, 67)
Alkylator	Cyclophosphamide	(72, 91)	(72)	(230, 236, 243–245)
	Ifosfamide	(246)		
	Melphalan	(235, 247)		(235)
	Peptichemio	(51)		
	Mitomycin-C	(62)		
	Cisplatin	(47, 59, 212)	(47, 248)	(56, 65, 249)
	Carboplatin	(47, 60, 103)	(47, 59)	
	Oxaliplatin	(59, 61)	(59, 61, 87, 250)	(66)
	B-85-0040	(251)		(251)
	Nedaplatin	(252)	(252)	
Nitrosourea	Cystemustine	(57)		(57)
Cytokines	rHu-interferon α	(253)		(253)
	rMu-interferon γ	(253)		(253)
	Interferon β	(98)	(98)	(98)
	Tumor necrosis factor α	(254)		(255)
	Interleukin-2	(50)		(256)
CDKI	Seliciclib	(49)	(49)	(94)
Cox-2 inhibitor	Celecoxib	(96)		(96)
VEGF inhibitor	TNP-470	(97)	(97)	(97)
	SU 1498			(100)
	BB2516			(100)

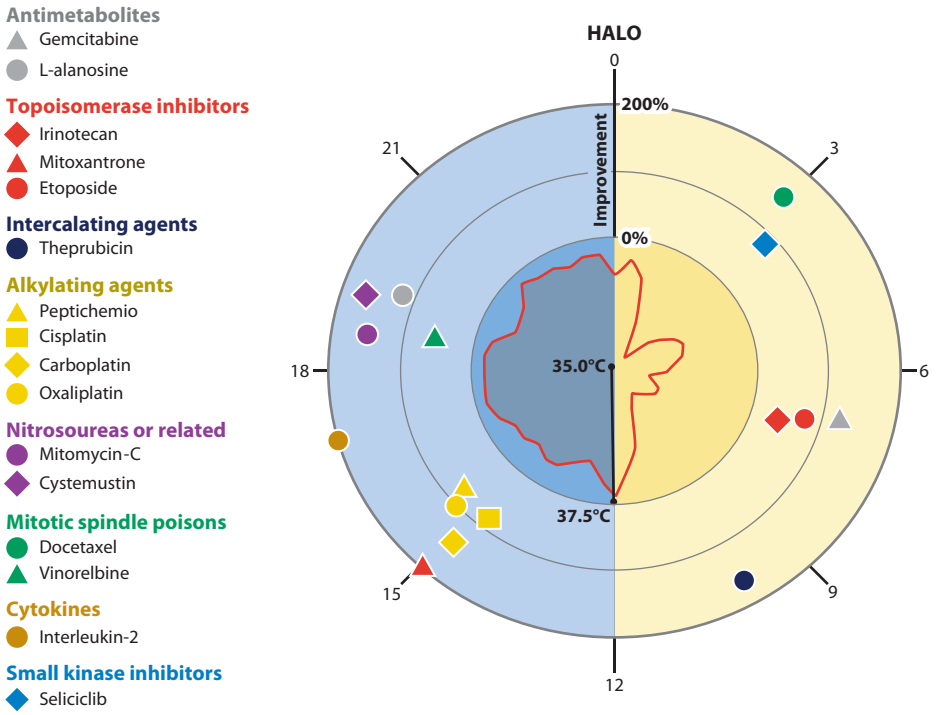


Figure 3

Relevance of circadian timing for the tolerability of anticancer drugs. Circadian timing associated with best tolerability, in ZT (or hours after light onset, ranging from 0 to 24) and relative magnitude of survival benefit from optimal to worst timing, ranging from 0 to 200%. The diagram illustrates chronotolerance for 16 anticancer drugs studied in our laboratory in male B6D2F1 mice, synchronized with LD12:12. The average circadian rhythm in body temperature is shown in the internal circle and provides a CTS biomarker, an endogenous reference for optimal drug timing (see **Table 1** for corresponding references).

the other hand, nonrhythmic *Cyp3a13*, rhythmic *Cyp2b10*, and possibly *Cyp2c29* participate in the circadian tolerability of cyclophosphamide (29, 72). In contrast, 5-FU and gemcitabine, whose toxicity also depends upon circadian timing, undergo rapid liver catabolism through dihydropyrimidine dehydrogenase (DPYD) and cytidine deaminase activities, respectively (73–75).

The carboxylesterases *Ces1* and *Ces2* are rhythmically controlled both by the circadian clock and by clock-controlled *Dbp*, *Tef*, and *Hlf* in the liver and gastrointestinal tract (29, 76). *Ces1* and *Ces2* circadian expression can account for the increased biotransformation of irinotecan into SN-38 during the light (rest) span of male ICR mice (77). *Dpyd* mRNA expression and activity display significant rhythms in the liver of male B6D2F1 mice, with an ~15-h time lag between their peaks. Peak DPYD activity occurs near the middle of the light span, when the animals rest (78). All the enzymatic activities that generate the cytotoxic forms of 5-FU, such as orotate phosphoribosyltransferase, uridine phosphorylase, and deoxythymidine kinase, are highest during the dark (activity) span of rats or mice, when 5-FU is most toxic to healthy tissues (23).

Rhythmic phase II detoxification by reduced glutathione (GSH) is a critical determinant of the toxicities of platinum complexes and other cytostatics. Liver and jejunum GSH contents are approximately threefold higher in the second half of the dark span in mice and rats compared with mid-light, and the pharmacologic suppression of GSH synthesis with buthionine

GSH: reduced glutathione

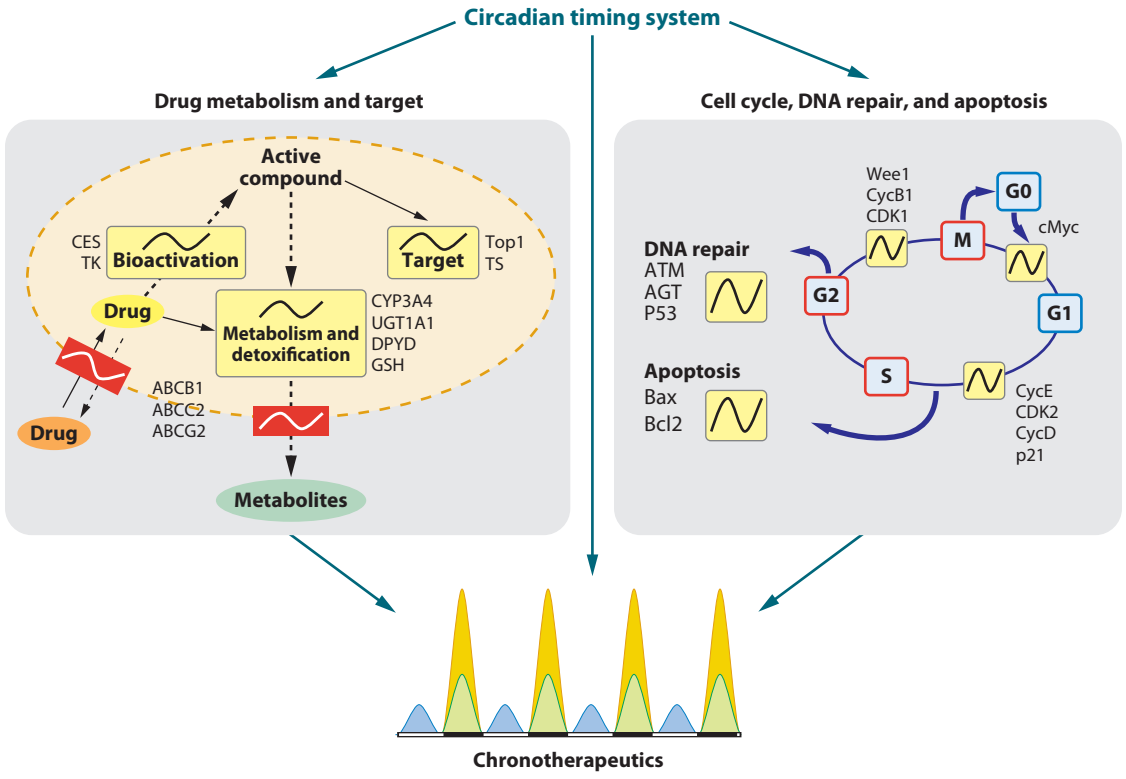


Figure 4

Main cellular determinants of cancer chronotherapeutics. The CTS (*top*) determines the optimal circadian timing of anticancer medications (*bottom*). The CTS controls drug transport, bioactivation, detoxification, metabolism, targets, and elimination, which account for the chronopharmacology of anticancer agents at cellular, tissue, and whole organism levels (*left*). The CTS also regulates several cell-cycle-related events that gate G1/S or G2/M transitions, as well as DNA repair and apoptosis, which account for the chronopharmacodynamics of anticancer drugs (*right*). The relations between chronopharmacokinetics and chronopharmacodynamics help construct optimal chronomodulated drug delivery schedules, with proper parameters.

sulfoximide profoundly alters the chronotolerance pattern of cisplatin and oxaliplatin in mice (79, 80).

UGT1A catalyzes the detoxification of seliciclib, irinotecan, and SN-38. Highest UGT activity is reported during the dark (activity) span of rats (70). However, mean and circadian expression of *Ugt1a1* differ according to species, strain, and gender, with relevant consequences for irinotecan chronotolerance (81, 82). Circadian clocks control the transcription of ATP-binding cassette family members, including *Abcb1a* and *Abcb1b (Mdr1)*, *Abcc2* (*Mrp2*), and *Abcb4* (*Mdr2*) in mouse liver and intestine (29, 69, 83–85). The *Abcb1a* and *Abcb1b* mRNA rhythms translate into a 30% increase of permeability glycoprotein (*P-gp*) activity at ZT17–19 compared with ZT5–7 in rat jejunum and ileum (B. Lemmer & A. Okyar, unpublished data).

The multiple circadian controls of drug absorption, distribution, metabolism, and elimination (ADME) account for dosing time dependencies in the pharmacokinetics of 17 anticancer drugs of all classes in mice, rats, and even pigs (**Table 1**). Even the continuous delivery of 5-FU by an implanted pellet results in circadian changes in plasma drug concentrations, with high values at daytime, during the rest span of the mice (86). Circadian timing mostly affects the initial distribution phase (C_{max}, $t_{1/2\alpha}$, Vdi), the area under the concentration x time curve (AUC), and the plasma

P-gp: permeability glycoprotein

TS: thymidilate synthetase

Top: topoisomerase

clearance of anticancer agents. High circadian drug exposure of healthy tissues, based on plasma PK analysis, is related to the high circadian toxicity of methotrexate, mitoxantrone, interferon-α, and the antiangiogenic agent TNP-470 in mice (**Table 1**). However, no consistent relationships are found between blood chronopharmacokinetics and chronotolerance for irinotecan, cyclophosphamide, cisplatin, carboplatin, oxaliplatin, interferon β, or seliciclib. For instance, highest Vdi and elimination are observed in mice dosed with carboplatin at ZT8 and with oxaliplatin at ZT16, despite both drugs being least toxic at ZT16 (87). Highest platinum content is found in 12/18 tissues 24 h after a single dose of oxaliplatin at ZT8, when this drug is most toxic (61). However, no consistent relationship is found in the tissues of mice treated at ZT 24, when the toxicity of oxaliplatin is intermediate, or at ZT16, when it is least toxic (61). The plasma and liver pharmacokinetics of orally given seliciclib differ significantly according to circadian timing. Seliciclib AUC is 25% greater at ZT3 than at ZT19 in plasma, and 80% less at ZT3 than at ZT19 in liver, when the drug produces the fewest liver alterations (49). Taken together, the results both emphasize and qualify the relevance of chronopharmacokinetics as mechanisms of chronotolerance for anticancer medications.

Circadian Control of Cell Cycle, DNA Repair, Apoptosis, and Molecular Targets

Cell cycle events are coordinated along the 24-h period in healthy bone marrow, gut, and skin, three frequent targets for the toxicity of cancer treatments (24). Proportions of S- and G2/M-phase cells increase by ~50% in the second half of darkness, whereas G0-G1 cells predominate during light in the total bone marrow of male B6D2F1 mice (33, 58). In this tissue, BCL2 protein expression triples over the 24-h period, with a maximum at early light. An opposite pattern characterizes proapoptotic BAX, with a fivefold 24-h change and a peak at ZT15 (33). The temporary arrest of cycling cells in G0-G1, the high BCL2, and low BAX expressions during the light span when mice rest help explain the best circadian timing for the tolerability of 5-FU, gemcitabine, irinotecan, and docetaxel in male B6D2F1 mice (48, 56, 58, 67, 73). However, the circadian control of drug metabolism and detoxification also profoundly modifies the cellular exposure to these medications, whose molecular targets are usually clock regulated (77, 88). For instance, the increased detoxification of 5-FU during the light span results from the circadian peak in DPYD activity in liver and other healthy cells (73, 78). It thus adds up to the reduced proportions of S-phase cells in bone marrow, gut, and skin, as a mechanism for improved circadian tolerability (73). Both transcription and activity of thymidilate synthetase (TS), which provide the unique de novo source of thymidilate, are linked to early S-phase in proliferating tissues (89). Consistently, bone marrow TS activity peaks near mid-dark in coincidence with the greatest hematologic toxicity of 5-FU in female CD2F1 mice (88).

Whereas circadian Phase I and II metabolism partly determines irinotecan pharmacology over the 24-h period, topoisomerase 1 (Top1), the main protein target of this drug is mostly at work during late S-phase. Thus, the circadian gating of the cell cycle and possibly the direct control of *Top1* by the molecular clock also contribute to the better hematologic tolerability of irinotecan during the rest span of male ICR mice (77, 90).

The Role of Molecular Clocks and Clock-Controlled Pathways in Chronotolerance

The CLOCK-BMAL1 transactivation complex represses cyclophosphamide toxicity mechanisms and partly determines the chronotolerance pattern of this drug. Cyclophosphamide was best tolerated at ZT10-ZT14 in two studies carried out ~30 years apart in female wt C57Bl6 mice (72, 91).

Cyclophosphamide tolerability is worse in *Clock*$^{m/m}$ and *Bmal1*$^{-/-}$ mice, whereas it is improved in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice (72). In these three strains with a genetically disrupted molecular clock, chronotolerance patterns of cyclophosphamide are blunted (72). Circadian pharmacokinetics result in greatest formation of *4-OH*-cyclophosphamide and dechloroethylcyclophosphamide in wt mice dosed at ZT2, when the drug is most toxic. *Clock*$^{m/m}$ increases formation of the bioactive *4-OH*-cyclophosphamide and profoundly modifies cyclophosphamide metabolism (72). The tolerability of cyclophosphamide, mitoxantrone, vincristine, and methotrexate is best near ZT12, when *Dbp*, *Tef*, and *Hlf* expressions are high (29, 92). Whereas vincristine and methotrexate show no significant differences in toxicity between wt and triple *Dbp*, *Tef*, *Hlf* knockout mice; both mitoxantrone and cyclophosphamide are much more harmful in PAR bZip-deficient as compared with PAR bZip-proficient animals (29). Clock-controlled PAR bZip transcription factors play a critical role in the detoxification of anticancer drugs whose metabolisms involve carboxylesterases (CES), sulfotransferase, UGT1A, glutathione S-transferase, and ABC transporters such as P-gp and breast cancer resistance protein (BCRP) that are responsible for the intestinal and biliary secretions of several anticancer drugs (29).

ABC transporter: ATP-binding cassette transporter

GOS: Glasgow osteosarcoma

The Relevance of Drug Timing for Treatment Efficacy

Circadian timing also critically affects antitumor efficacy of 28 anticancer medications, including cytostatics, antiangiogenic agents, and cell cycle or Cox2 inhibitors in rodents with various kinds of malignancies (**Table 1**). The demonstration of chronoefficacy is based on the administration of a single agent for several days or weeks and/or its combination with up to four other drugs at stipulated circadian times (93). Appropriately circadian-timed and dosed chemotherapy with one or several drugs at least halves tumor growth rate and/or significantly increases life span in tumor-bearing mice (56, 66, 94). Circadian timing also largely modifies the efficacy of anticancer agents against human cancer cells from breast (MCF-7, ZR-75–30, and MDA-MB-468) or colon (HCT116) transplanted into nude mice (95, 96).

Strikingly, the circadian pattern in chronoefficacy usually coincides with that in chronotolerance (**Table 1**). This is true for cytostatics, interferons, antiangiogenic agents, and cell cycle inhibitors, as well as for combination chemotherapy, such as irinotecan-oxaliplatin, gemcitabine-cisplatin, and docetaxel-doxorubicin, three widely used clinical regimens (56, 66, 67, 97, 98). Experimental chronotherapeutics thus strongly supports circadian timing as a relevant method for improving anticancer treatments.

The chronoefficacy of anticancer medications can partly result from circadian changes in tumor drug uptake, as shown for methotrexate in sarcoma-bearing rats, interferon β in mice with B16 melanoma, and seliciclib in mice with Glasgow osteosarcoma (GOS) (94, 98, 99) (M. Hassan, E. Filipski, & F. Lévi, unpublished data). Chronoefficacy can also stem from the circadian control of drug pharmacodynamics in tumors, as shown for cell cycle phase distribution, related protein targets, such as TS for 5-FU and Top1 for irinotecan and receptors, such as interferon-α/β receptors (88, 90, 98). Vascular endothelial growth factor is also produced rhythmically in slow-growing mouse sarcoma-180 with a maximum near ZT2, when the antitumor efficacy of three antiangiogenic agents doubles compared with an administration at ZT14 (100).

However, circadian disruption frequently adds to cell cycle disruption as a hallmark of cancer, at least in rapidly growing malignancies and at an advanced stage of tumor evolution (14, 24, 32, 34, 93, 94). Clock gene transcription is no longer circadian in advanced GOS or pancreatic adenocarcinoma P03 (14, 94) (X. M. Li, F. Delaunay, S. Dulong, B. Claustrat, S. Zampera, et al., submitted manuscript). No circadian organization is found for S-phase cells in GOS or mammary carcinoma MA13C, for BCL2 protein expression in MA13C, or for GSH content in P03 (14, 33,

93, 94). Nevertheless, chronoefficacy remains robust in these experimental tumors (56, 58, 93, 94), possibly because (*a*) the CTS of the host determines the chronoefficacy of anticancer medications, and/or (*b*) an adequate resetting of tumor circadian clocks by anticancer medications critically contributes to their efficacy.

The Circadian Timing System as a Target for Anticancer Treatments

Treatment-induced circadian disruption. Biomarkers of CTS coordination such as rest-activity and core body temperature can be severely disrupted by anticancer agents of any pharmacologic class (**Table 2**). This is also the case for rhythms in urinary excretion, blood cell counts, and other circadian biomarkers of chemotherapy toxicities. Anticancer agents also impair molecular circadian clocks in the SCN, liver, adrenals, or other peripheral organs of mice and in cell cultures (49, 101). **Table 2** shows the disruption of host circadian rhythms for 12 anticancer medications in experimental models.

The extent of alterations and the recovery dynamics of rest-activity and body temperature rhythms vary as a function of both dose and circadian timing, as shown for vinorelbine and gemcitabine (74, 102). For instance, a single therapeutic high dose of gemcitabine mildly alters both SCN biomarkers if the drug is given at ZT11, but it markedly suppresses them if it is administered at ZT23, when it is most hematotoxic (**Figure 5**) (56, 74). Circadian timing determines the extent and duration of SCN disruption produced by a single therapeutic dose of irinotecan, oxaliplatin, vinorelbine, interferon-α, or seliciclib (74, 101, 102) (**Table 2**). Conversely, treatment at the circadian time associated with fewest toxicities best spares the CTS, irrespective of the underlying toxicity mechanisms or target tissues. Similar findings characterize circadian biomarkers of tissue toxicity. Thus, blood cell count rhythms are maintained or suppressed in mice dosed with theprubicin and carboplatin, near their respective best or worst circadian timing (46, 53, 103).

Circadian disruption also affects the molecular clocks in the central pacemaker and in peripheral tissues. The circadian patterns in mRNA expression of *Per1*, *Per2*, *Per3*, and *Bmal1* in the SCN are ablated in mice receiving interferon-α at ZT12 and maintained in those treated at ZT0 (101). Mice on interferon-α at ZT0 also maintain near normal *Per1* mRNA rhythm in liver and in adrenals. Conversely, *Per1* expression rhythm is damped and advanced by ∼4 h in the mice treated at ZT12 (101). Inappropriately timed anticancer agents can modify circadian clock amplitude and phase in peripheral organs and prevent the predictability of internal circadian timing (46, 74, 101–104). Constant-rate infusion of 5-FU with an osmotic minipump attenuates the circadian rhythms in *Per1* and *Per2* mRNA in both SCN and liver of mice (104). Seliciclib, a cyclin-dependent kinase (CDK) inhibitor, either profoundly disrupts *Rev-erbα*, *Per2*, and *Bmal1* transcription rhythms at ZT3 or only dampens their patterns at ZT19 in mouse liver (**Figure 5**) (49). The circadian disruption of the liver clock at ZT3 is selectively associated with liver toxicity (49). Dose-dependent disruption of circadian clocks also results from in vitro exposure of synchronized NIH3T3 fibroblasts or Period2-Luciferase expressing C6 glioma cells to 5-FU (104).

Circadian induction by anticancer agents. Spontaneous or imposed rhythmic patterns in corticosterone, body temperature, or feeding entrain relevant cell cycle and pharmacology determinants over the 24-h period in premalignant tissues or in tumors and slow down cancer processes (14, 90) (X. M. Li, F. Delaunay, S. Dulong, B. Claustrat, S. Zampera, et al., submitted manuscript). DNA repair elicited by γ-radiations and possibly also by anticancer drugs resets free-running host circadian clocks via ATM-mediated damage signaling (105). However, tumors frequently escape from circadian coordination. Thus, no circadian expression pattern is found for *Per2*, *Bmal1*, and

Table 2 Effects of anticancer drugs on the circadian timing system in experimental models

Class of agent	Name	Test system	Disrupted rhythm	Role of circadian timing	Reference
Antimetabolite	5-fluorouracil	Mouse (male ICR)	Locomotor activity *Per 1*, *2* in liver & SCN	NA	(104)
		Mouse NIH3T3 (culture)	*Per 1*, *2*	NA	
	L-alanosine	Mouse (male B6D2F1)	Rest-activity Body temperature	NA	(55)
	Gemcitabine	Mouse (male B6D2F1)	Rest-activity Body temperature	Yes	(74)
Top1 inhibitor	Irinotecan	Mouse (male B6D2F1)	Rest-activity Body temperature	Yes	C. Ahowesso & F. Lévi (unpublished data)
Intercalators	Theprubicin	Mouse (male B6D2F1)	Blood cell rhythms	Yes	(46)
Alkylators	Cisplatin	Rat (female F344)	Body temperature Urinary rhythms	Yes	(257)
	Oxaliplatin	Mouse (male B6D2F1)	Rest-activity Body temperature	Yes	E. Filipski & F. Lévi (unpublished data)
	Carboplatin	Mouse (male ICR)	Blood cell rhythms	Yes	(103)
Mitosis inhibitor	Vinorelbine	Mouse (male B6D2F1)	Rest-activity Body temperature	Yes	(102)
Radiation	γ-radiation	Mouse (male C57BL/6J)	Locomotor activity	Yes	(105)
		Rat fibroblasts	*Per 1*, *Per 2*, *Clock*, *BMal1*	Yes	
Cell cycle inhibitor	Seliciclib	Mouse (male B6D2F1)	Rest-activity Body temperature *Rev-erba*, *Per 2*, *BMal1* in liver	Yes	E. Filipski & F. Lévi (unpublished data) (49)
Cytokine	Interferon-α	Mouse (male ICR)	Locomotor activity Body temperature *Per 1*, *2*, *3*; *Clock*; *BMal1* in SCN, liver, adrenals	Yes	(258) (101)
		HepG2 (culture)	*Clock* & *Bmal1* mRNA & protein	NA	(259)

*Rev-erb*α in advanced GOS or pancreatic adenocarcinoma P03 (14, 94) (X.M. Li, F. Delaunay, S. Dulong, B. Claustrat, S. Zampera, et al., submitted manuscript). An induction of near normal circadian patterns in clock gene transcription is produced in GOS, with five daily oral administrations of seliciclib at ZT3, but not at ZT19. Tumor clock induction by seliciclib at ZT3 nearly doubles the antitumor efficacy of seliciclib as compared with ZT19 treatment. Mechanisms involve the transient inhibition of casein kinase I δ/ε, an enzyme that regulates the intrinsic period of the circadian clock itself and translates into differential expressions of clock-controlled genes *c-Myc* and *Wee1* (94).

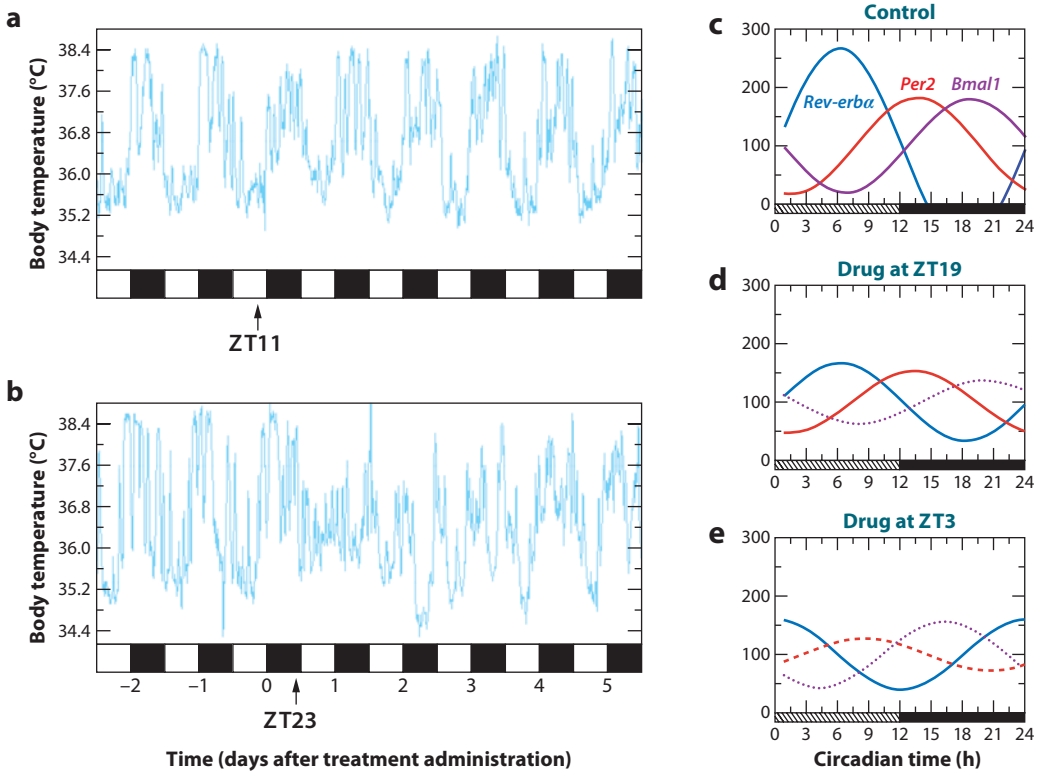


Figure 5

Circadian disruption resulting from anticancer medications. (*Left*) Effect of a single dose of gemcitabine (400 mg/kg iv) on the circadian rhythm in intraperitoneal temperature recorded via telemetry. The drug administration (vertical arrow) is at (*a*) ZT11 or (*b*) ZT23 (disruption), when it also achieves best or worst hematologic tolerability, respectively. Adapted with permission from Reference 74. (*Right*) Effects of five daily doses of seliciclib (300 mg/kg/d po) on the liver molecular clock as estimated with transcriptional rhythms in *Rev-erbα* (blue line), *Per2* (red line), and *Bmal1* (purple line). Solid lines correspond to statistically validated 24-h rhythms. (*c*) Control liver clock. Drug administration is at (*d*) ZT19 or (*e*) ZT3 (disruption), when it achieves best or worst hepatic tolerability, respectively. Adapted with permission from Reference 49. Both studies involve male B6D2F1 mice synchronized by LD12:12 (alternation of open and dark boxes).

Implications for cancer treatments. Most anticancer drugs that disrupt SCN biomarkers are moderately taken up in brain tissue, yet no specific information about SCN drug uptake exists (106, 107). Anticancer therapy can induce the release of several cytokines or growth factors that can also modify the CTS (108, 109). Transforming growth factor α, epidermal growth factor, CLC, and PK-2 suppress SCN biomarkers following their infusion in the third ventricle of mice or hamsters (20–22). These peptides can penetrate the brain from the systemic circulation (110–112). IL-6 induces *hPer1* in HU-H7 hepatoma cells (113). Tumor necrosis factor-α (TNF-α) disrupts circadian clocks and *Dbp*, *Tef*, and *Hlf* in the liver and in the SCN of mice, as well as *Per1*, *Per2*, and *Per3* circadian expression in cultured fibroblasts (114). Tumor necrosis factor-α inhibits CLOCK-BMAL1-induced activation of E-box regulatory-element-dependent clock gene promoters (114). Thus, the release of cytokines during toxic processes can disrupt host circadian clocks. In turn, circadian disruption accelerates malignant growth, as shown in mice with *Per2* suppression, SCN ablation, or chronic jet lag exposure (9, 10, 115). Direct effects on the clock

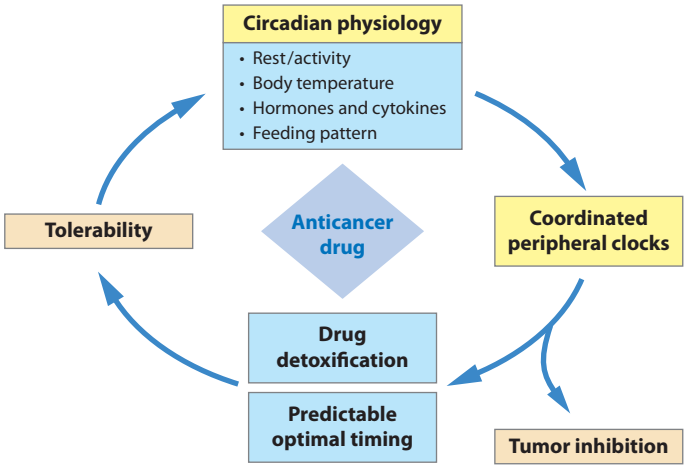


Figure 6

Scheme integrating the CTS in the therapeutic objectives of anticancer treatments. The adequate circadian timing of properly dosed anticancer medications ensures good treatment tolerability. In turn, adequate circadian physiology is maintained, molecular clocks in target tissues remain synchronized, drug metabolism and detoxification occur smoothly, and the optimal circadian timing remains predictable for subsequent drug administrations. In addition, the proper functioning of the CTS synergizes anticancer drugs with regard to their antitumor efficacy. In contrast, the elicitation of severe toxicities, through improperly timed and/or dosed medications, disrupts circadian physiology, desynchronizes molecular clocks, alters drug metabolism and detoxification, impairs the predictability of optimal circadian timing, and alleviates CTS control on tumor progression.

mechanisms or signaling pathways also have to be considered, especially for noncytotoxic agents such as seliciclib (94, 116).

An uncoupling within the host CTS could further increase the susceptibility of the organism to anticancer drugs, through an alteration of the fine-tuned circadian coordination of detoxification pathways (29). Circadian disruption then impairs the dynamics of detoxification and retards the recovery from toxicity at tissue and central levels, in good agreement with experimental chronotolerance data. High systemic toxicity of anticancer drugs seems to correlate with circadian disruption, and circadian disruption accelerates cancer progression (9, 10, 115). Cancer chronotherapeutics could then aim for the minimization of host clock disruption to prevent toxicities and the induction of tumor clocks to inhibit cancer progression (**Figure 6**).

STANDARDIZED CLINICAL CANCER CHRONOTHERAPEUTICS

Experimental chronotherapeutics suggest that some anticancer treatments are expected to be best tolerated and most effective at odd hours in cancer patients. This qualification is handled by dedicated drug delivery technologies, which further allow the design of novel circadian chronomodulated schedules. In turn, these schedules undergo validation steps for pharmacokinetics, clinical tolerability, and efficacy.

Drug Delivery Technology for Cancer Chronotherapeutics

The concept and the industrial development of nonimplantable multichannel programmable pumps have fostered the clinical development of cancer chronotherapeutics. Multiple circadian

ChronoFLO: chronomodulated delivery of 5-FU, leucovorin, and oxaliplatin

infusional schedules are jointly administered to nonhospitalized patients, with minimal or no medical or nursing intervention. The advent of IntelliJect™ with four 30-ml reservoirs enabled the development of the first combination schedule of 5-FU-leucovorin-oxaliplatin and led to the initial demonstration of the safety and efficacy of this three-drug chemotherapy given according to a circadian chronomodulated delivery schedule, several years before the registration of oxaliplatin (17).

The approval of IntelliJect in the European Union and in North America allowed its use for the routine chemotherapy of cancer patients as well for the evaluation of standardized chronomodulated infusions of 5-FU-leucovorin-oxaliplatin (chronoFLO) within international clinical trials. Mélodie™, a second generation of electronically engineered four-channel programmable pumps, represents considerable technological progress, through increased energy autonomy, flexible reservoir capacity, rapid programming of any delivery schedule, computer storage of treatment protocols and patient data, as well as actual drug delivery reports for each treatment course. The infusional pressure of this pump allows the administration of irinotecan-5-FU-oxaliplatin in a European trial of three-drug chronomodulated infusions into the hepatic artery (117) (OPTILIV07: EUDRACT number: 2007-004632-24, ClinicalTrials.gov, identifier: NCT00852228). Preprogrammed, single-use, and elastomeric pumps with multiple electronic valves (CIP™) represent a recent concept of versatile multichannel chronotherapeutic drug delivery in ambulatory settings (118). Albeit lighter, ready-to-use, and disposable, the CIP requires modifications of drug delivery profiles to be performed using dedicated technology systems before use. This European Union–approved system is currently used both in daily oncology practice and in multicenter clinical trials (M. Pirovano & C. Garufi, personal communication). Conventional chemotherapy protocols only consider drug doses, duration, and frequency of infusions. As a result, treatment times vary among and within patients. However, 85% of the cancer treatments are administered from 9:00 a.m. to 5:00 p.m., that is, over only a third of the day span (119). In contrast, circadian chronomodulated schedules stipulate the time courses and parameters of the delivery profile for each anticancer medication over the 24-h period to achieve the best therapeutic index. This includes times of onset and offset of infusion and variation of flow rate, ranging from constant to sinusoidal or gradually increasing or decreasing.

Orally formulated anticancer medications are also amenable to chronotherapeutic delivery. Oral fluoropyrimidines seem to be best tolerated with systemic drug exposure at night (120–122). However, optimal chronotherapeutic delivery at night when the patient is sleeping requires adaptive drug delivery technologies. Novel oral pulsatile drug delivery systems release active drug principles after a predetermined lag time following ingestion and have proven their clinical relevance for chronotherapeutics (123, 124). Such systems could critically improve the safety and efficacy of orally dosed anticancer medications through circadian optimization of drug exposure.

Spontaneous or Imposed Circadian Control of Anticancer Drug Pharmacokinetics

Circadian timing significantly influences the plasma and/or urinary pharmacokinetics of intravenously administered 5-fluorouracil, methotrexate, doxorubicin, epirubicin, and cisplatin in cancer patients (125–130). This is also the case for orally administered busulfan, 6-mercaptopurine, and tegafur/uracil (120, 131–134). Continuous intravenous infusion results in 24-h changes in plasma concentrations for 5-FU (over 1–14 days), doxorubicin (over 2–42 days), and or vindesine (over 4 days) despite respective half-lives of 20 min, several hours, and 24 h (73, 135–138). The highest average plasma concentrations of 5-FU are found between 2:00 a.m. and 6:00 a.m. despite constant-rate infusion of this drug in 9 of 11 studies involving a total of 270 patients (23). The

duration of infusion, as well as patient gender, genotype, lifestyle, disease stage, and other drugs given concurrently can modify the average circadian pattern. Thus, both the mean and circadian amplitude of plasma clearance are halved in women as compared with men on a 2-day constant-rate infusion of 5-FU (139).

Circadian pharmacokinetics of anticancer drugs is found in children and in adults and deserves exploration in elderly cancer patients (140). Interpatient variability may mask chronopharmacokinetics, as shown in individual studies for oral methotrexate or 6-mercaptopurine, intravenous carboplatin, and continuous 5-FU or etoposide infusions (141–144). However, circadian changes of up to fivefold frequently characterize drug exposure in individual cancer patients. The mechanisms that drive human circadian pharmacokinetics partly match those already discussed in experimental models. Yet relevant dynamic biomarkers of drug metabolism and detoxification are usually lacking in cancer patients, except for the urinary excretion pattern of 6-β-OH-cortisol, which has been proposed as a biomarker of human CYP3A activity (145).

Chronomodulated infusions not only optimize drug exposure parameters according to circadian timing of peak delivery but also reduce interpatient variability. This is illustrated in two studies involving 27 patients with metastatic colorectal cancer receiving infusional 5-FU-leucovorin-oxaliplatin for 4 or 5 days (73, 87, 135). Total AUC of 5-FU varied fivefold among patients on constant-rate infusion and less than 0.7-fold in those on chronoFLO, despite the same dose (per square meter) being infused (73, 135). The drastic reduction in PK variability requires both chronomodulated infusion and the assignment of peak flow rate at 4:00 a.m. A highly statistically significant increase in inter- and intrapatient variability characterizes the Cmax of 5-FU if the drug delivery rate peaks at 1:00 p.m. or 7:00 p.m. (73, 135). Patients with a large regular circadian variation in the 5-FU plasma concentrations and a Cmax located at 4:00 a.m. display best tolerability (73, 87, 135). The estimated total and free platinum AUCs are significantly lowest and most variable in patients receiving chronomodulated oxaliplatin with peak delivery at 1:00 a.m., compared with those with peak infusion rate at 7:00 a.m. or 4:00 p.m. (87). Diffusion of oxaliplatin out of the plasma compartment is likely to be greatest in the late evening or early night hours, when peripheral vascular resistance, plasma proteins, and erythrocyte membrane microviscosity are lowest (23, 146). The patients receiving chronomodulated oxaliplatin with a peak at 4:00 p.m. also experience less diarrhea and less peripheral sensory neuropathy than those with peak delivery at 1:00 a.m. or 7:00 a.m. (87). The relations between the circadian control of 5-FU and oxaliplatin pharmacokinetics and treatment tolerability have been confirmed in subsequent large clinical trials (147–149).

The relation between pharmacokinetics and toxicity has also been investigated in a randomized study involving 31 cancer patients receiving irinotecan, whose terminal half-life is ~12 h. This agent was administered as a conventional 30-min infusion in the morning or as a chronomodulated infusion from 2:00 a.m. to 8:00 a.m., with peak delivery rate at 5:00 a.m., based on results from experimental studies (48, 66, 77). The interpatient variability of irinotecan and SN-38 exposure was largest in patients given a conventional 60-min infusion in the morning. Conversely, good reproducibility characterized the time curves of drug and metabolite concentrations in patients receiving the chronomodulated infusion, which also produced fewer episodes of severe diarrhea than conventional administration (**Figure 7**) (150).

Circadian Control of Cellular Determinants of Cancer Chronotherapeutics

Clock genes are expressed at mRNA and/or protein levels in bone marrow, peripheral blood mononuclear cells, circulating leukocytes, oral and colorectal mucosae, skin, heart, liver, lung, breast, ovary, endometrium, abdominal fat, and pineal gland of healthy subjects (151–160). Peak

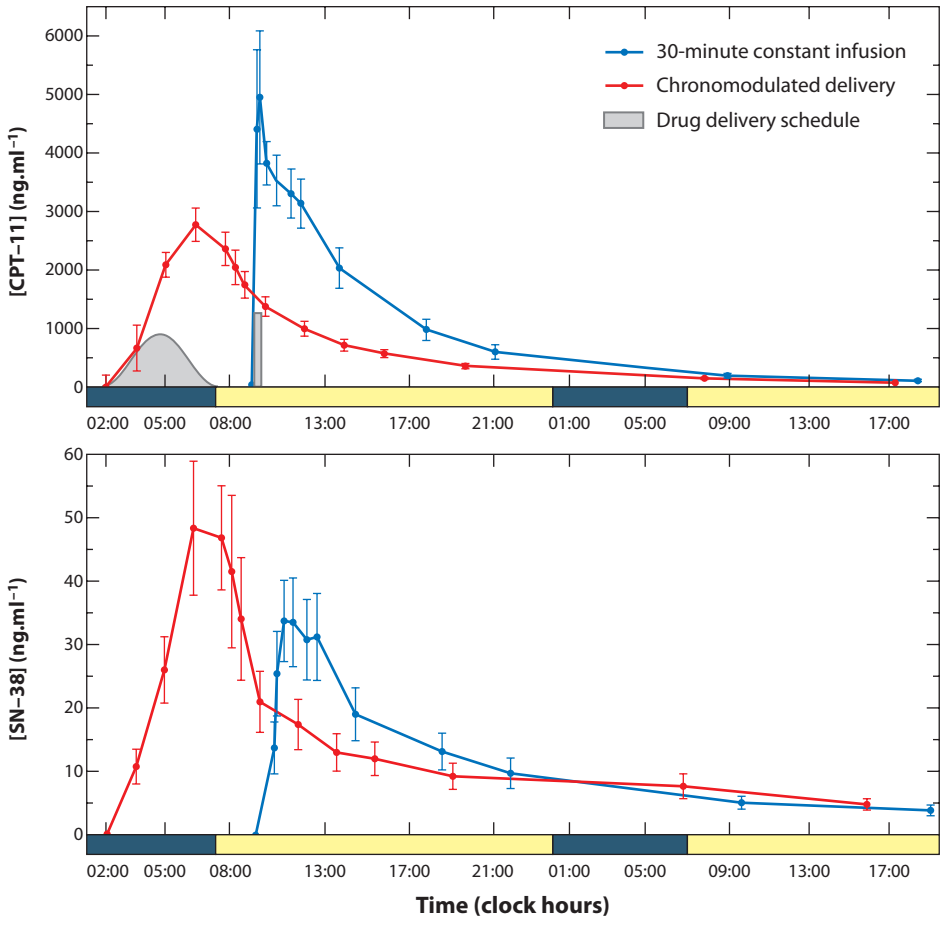


Figure 7

Relevance of circadian timing and chronomodulated delivery for irinotecan pharmacokinetics in cancer patients. Average time courses of plasma pharmacokinetics of irinotecan (CPT-11) and bioactive metabolite SN-38 following a conventional 30-min infusion near 10:00 a.m. or a 6-h chronomodulated infusion with peak flow rate at 5:00 a.m. Results are from a randomized study in 31 patients receiving a fixed dose of 350 mg/m^2 of irinotecan. Chronomodulated delivery (*a*) reduces mean C_{max} of irinotecan, from 5.53 to 2.91 μg.ml^{-1} ($p = 0.00012$) and its coefficient of variation from 37% to 17.4%, (*b*) increases the average metabolic ratio (AUC of SN-38:AUC of irinotecan) from 1.89% to 2.53% ($p = 0.02$), and (*c*) reduces the incidence of severe diarrhea from 22.2% to 6% and that of severe asthenia from 44% to 23.5% compared with conventional delivery. Adapted from Reference 189.

mRNA is usually highest in the morning for *hPer1* and *hPer2* and in the evening for *hBmal1*, resulting in similar phase relationships between clock gene transcription patterns in humans and laboratory rodents (157, 160–165). Relevant clock-controlled genes for anticancer drug pharmacology are identified through human circadian transcriptome studies in oral mucosa, mammary epithelium, and adipose tissue (157, 161, 165). Depending on the tissue sampled, the microarray chip used, and the sampling frequency, 4% to 25% of the human genome display circadian variations (157, 161, 165). Gene ontology analysis reveals that most oscillating genes in these differentiated human tissues regulate gene transcription, cell cycle, or metabolism (157, 161, 165). In mononuclear cells of healthy subjects, both *Dpyd* mRNA and activity display a circadian rhythm,

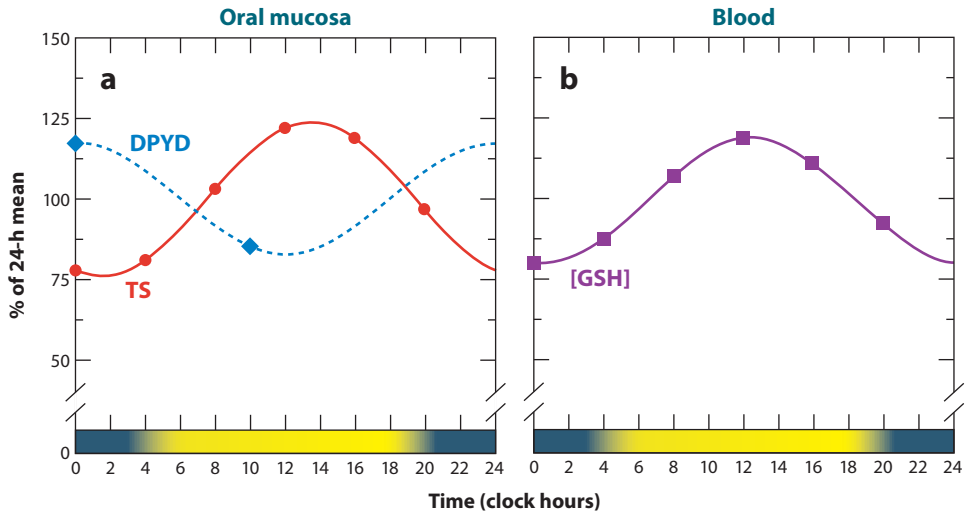


Figure 8

Examples of coordinated rhythmic detoxification and main drug targets in human tissues. (*Left*) Average 24-h sinusoidal estimate of DPYD and TS activities in human oral mucosa, a main target tissue for 5-FU toxicity. DPYD catabolizes 5-FU, whereas TS is the main pharmacologic target of this drug. Adapted with permission from References 213, 287. (*Right*) Average 24-h sinusoidal estimate of GSH concentration in the blood of patients with nasopharyngeal carcinoma. GSH plays an important role in the detoxification of platinum complexes and many other anticancer drugs. Adapted with permission from Reference 227.

with a maximum at night (166, 167). A similar trend for high values at midnight is found for DPYD activity in biopsies of oral mucosa (168). TS activity, but not mRNA, is also rhythmic in this tissue, with a maximum near 1:00 p.m. (163). The apparently opposite phases of DPYD and TS activities in oral mucosa support an increase in the tolerability of healthy tissues for nightly administration of 5-FU (**Figure 8**).

Plasma GSH concentrations vary by 30% along the 24-h cycle, with a maximum after midnight and superimposed peaks related to cystein intake during meals (169). Conversely, the highest GSH content occurs in the early morning hours and precedes by ~4 h the peak in S-phase cells in the bone marrow of healthy subjects (170). The protein O^6-alkylguanine-DNA alkyltransferase removes DNA adducts produced by nitrosoureas at guanine bases. The average O^6-alkylguanine-DNA alkyltransferase activity in circulating mononuclear cells increased by ~30% from noon to midnight in 12 healthy subjects (35). Anthracyclines, anthracenediones, and epipodophyllotoxins inhibit Top2a, resulting in DNA strand breaks (171). Top2a protein displayed a circadian rhythm with a mean rate of change of 40% and a maximum approximately 7:00 a.m. in the rectal crypt cells of 10 healthy subjects, in synchrony with the peak in DNA synthesis in the same tissue (172).

The cell cycle is synchronously coordinated by the CTS in skin, oral and rectal mucosae, and bone marrow of humans, as in rodents (170, 173). On average, twice as many S-phase myeloid and erythroid cells and bone marrow progenitors are found near 4:00 p.m. compared with midnight in humans (170). The peaks of S-phase cells in the skin and in the oral and rectal mucosae also occur between noon and 4:00 p.m. (173). The circadian organization of cell cycle phases is further demonstrated by consistent 24-h changes in protein markers of cell cycle checkpoints, such as cyclin E (G1/S), cyclin A (G2), and cyclin B1 (M) (162, 173). The rhythmic organization of p53 protein with a peak at 11:00 a.m. and that of *hBcl2* mRNA expression with a peak near 1:00 a.m.

DPYD: dihydropyrimidine dehydrogenase

further suggest a circadian control of apoptotic pathways in healthy human tissues, as in rodents (33) (G. Bjarnason, personal communication).

FOLFOX: conventional delivery schedule associating 5-FU, leucovorin, and oxaliplatin

Malignant processes can selectively alter some circadian rhythms that drive chronotherapeutic effects in cancer patients. Thus, no consistent circadian rhythm in *Dpyd* mRNA expression was found in a group of 10 patients with advanced gastrointestinal malignancies, a finding at variance with that reported in healthy subjects (166). However, DPYD activity is higher at night than in daytime in patients with gastrointestinal or nasopharyngeal cancer, as measured by enzymatic assays on mononuclear cells or the plasma dihydrouracil:uracil ratio. Thus, the circadian pattern in DPYD activity is similar to that found in healthy volunteers, a finding supporting posttranscriptional control (174, 175) (M. A. Barrat, F. Lévi, & G. Milano, unpublished data). Mean GSH concentration peaked near noon in the peripheral blood of a group of 16 Chinese patients with nasopharyngeal carcinoma (**Figure 8**) (175). In another group of 15 cancer patients, GSH content did not differ significantly between noon and midnight in bone marrow, yet the proportion of S-phase cells is highest at noon, as it is for healthy subjects (170). Iterative samplings of human cancers through repeat biopsies or cytology aspirations over a 24-h period reveal a circadian organization in some human cancers but not others, as shown for S- and M-phase cells, in individual patients with breast, ovarian, skin, head and neck, or lung cancer or non-Hodgkin's lymphoma (176, 177).

Most investigations in cancer patients, similar to those in healthy subjects, show intersubject variability in circadian waveform, described by periodic components, mesor, amplitude, and phase. However, rest-activity, body temperature, plasma cortisol, and melatonin and circulating blood cell counts, among many circadian biomarkers, display statistically validated and consistent 24-h rhythms in groups of patients with early- or late-stage cancer of the breast, lung, colon, prostate, ovary, or head and neck (176). These findings support the development of standardized cancer chronotherapeutics, with fixed circadian times of administration and fixed drug-delivery chronomodulated profiles for all patients.

Methodology and Results of Clinical Cancer Chronotherapeutics

Over 100 phase I and II clinical trials of cancer chronotherapeutics have involved patients with advanced or metastatic cancer of almost all origins according to a recent PubMed search. Randomized phase III trials have compared chronotherapeutic delivery to a control administration protocol without any time specification. However, patient or hospital convenience makes timing implicit in control treatments, despite the lack of any such stipulation (119, 178). Conversely, constant-rate infusion over at least 24 h eliminates any circadian timing hypothesis for drug administration. Experimental and clinical data show that a constant-rate infusion schedule lasting an integral multiple of 24 h constitutes an adequate control for proof of principle demonstration of cancer chronotherapeutics, if the pharmacologic properties of the drug permit it (**Figure 9**). This statement is also supported by several mathematical models that include circadian clocks (177, 179, 180).

Chronotolerance in cancer patients. A sequential cross-over design, with toxicity as the main endpoint, takes patients who display severe toxicity on constant-rate or conventional-delivery infusion and then subsequently receive chronotherapeutic administration. For instance, the administration of conventional 5-FU, leucovorin, and oxaliplatin (FOLFOX4) for high-risk colorectal cancer produced grade 3–4 neutropenia in 23 of 68 patients. They then received the same drug doses according to chronomodulated infusion (chronoFLOX1). The chronomodulated delivery schedule produced severe neutropenia in a single patient, despite no prophylactic rh-G-CSF being administered (**Table 3**) (M. Pirovano, personal communication).

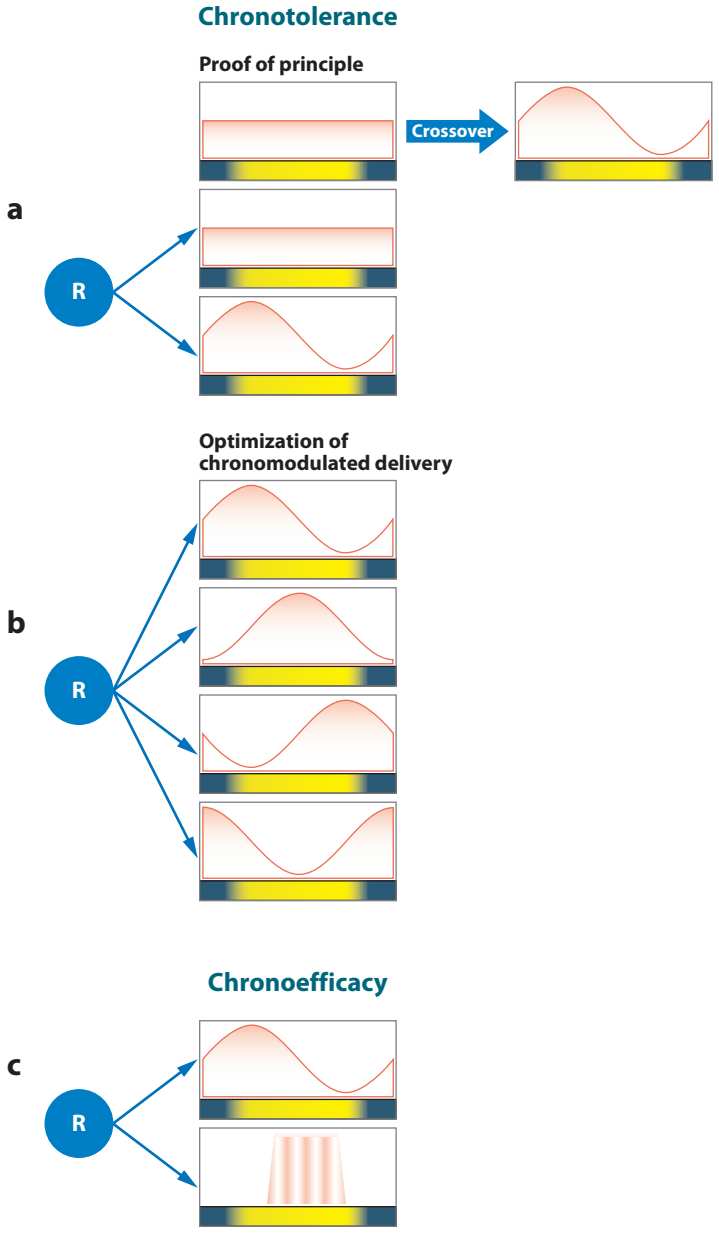


Figure 9

Examples of drug delivery infusion schedules and trial designs used for the clinical validation of standardized cancer chronotherapeutics. See results in **Table 3**. (*a*) Proof of principle of chronotolerance, through sequential or randomized trial design (R). (*b*) Optimization of chronomodulated drug delivery parameters and the relevance of circadian timing for peak drug delivery rate, with tolerability as the main endpoint, using sequential or randomized trial designs. (*c*) Validation of improved efficacy with randomized comparison with a conventional treatment, which is most often administered between 9:00 a.m. and 5:00 p.m.

Table 3 Main results of comparative clinical trials assessing the role of circadian timing of cancer chemotherapy in comparative trials in patients with colorectal or breast cancer. See text for details on drug delivery schedules.

Trial design	Compared schedules	No. of patients (cancer type)	Main endpoint(s)	Main results	Reference
Cross-over	FOLFOX4 → ChronoFLOX1	68 → 23 (Colorectal)	Gr 3–4 neutropenia	FOLFOX-4, 33.8% ChronoFLO, 4.3%	M. Pirovano, personal communication
Randomized	ChronoFLO5 vs constant rate (flat)	92 (Trial 1, colorectal)	Tolerability Tumor response	(Gr 3–4 mucositis) Chrono, 18% Flat, 89% (Tumor response rate) Flat, 32% Chrono, 53%	(149)
		186 (Trial 2, colorectal)	Tolerability Tumor response	(Gr 3–4 mucositis) Chrono, 14% Flat, 76% (Gr 2–3 sensory neuropathy) Chrono, 16% (Tumor response rate) Flat, 31% Chrono, 51% Flat, 29%	(148)
Time-finding	Eight chronoFLO4 lagged by 3 h	114 (Colorectal)	Tolerability	(Grade 3–4 toxicities) Peak drug delivery: at 4:00 a.m. for 5-FU-LV & at 4:00 p.m. for oxaliplatin, 16.7%; at 4:00 p.m. for 5-FU-LV & 4:00 a.m. for oxaliplatin, 80%	(147)
	Eight chronoVRL lagged by 3 h and fixed chrono5-FU	90 (Breast)	Tolerability	(Leucopenia) Significantly least if peak delivery rate of VRL near 5:00 p.m.	(181)
Randomized	ChronoFLO4 vs FOLFOX2 near MTD	564 (Trial 3, colorectal)	Survival	Similar overall survival. Hazard ratio: Worsened by 38% in females on chrono Improved by 25% in males on chrono	(183)
Randomized	ChronoFLO vs conventional delivery	842 (Meta-analysis of Trials 1–3, colorectal)	Survival per schedule according to gender	Similar overall survival. Hazard ratio (Cox): Worsened by 23% in females on chrono Improved by 23% in males on chrono	(184)

MTD, maximal tolerated dose.

Most multicenter trial designs involve randomized comparisons of a validated chronotherapeutic schedule with constant-rate infusions using the same initial doses over the same treatment duration. Experiments in male mice identified the times of least toxicity near mid-activity for oxaliplatin and near mid-rest for 5-FU (23). These circadian times were extrapolated to cancer patients, with the chronomodulated schedule combining the daily delivery of oxaliplatin over 11.5 h with peak flow rate at 4:00 p.m. and that of 5-FU-leucovorin over 11.5 h with peak flow rate at 4:00 a.m. for 5 consecutive days (chronoFLO5). The other cohort of patients received the same doses of the same three drugs, at a constant rate over the same 5-day span. In two international randomized phase III trials involving 278 patients with metastatic colorectal cancer, chronomodulated delivery reduced the incidence of grade 3–4 mucositis by fivefold and halved the incidence of peripheral sensory neuropathy (**Table 3**) (148, 149). The largest trial also reported a threefold reduction in the rate of hospitalizations for toxic events with chronomodulated infusions (148).

A subsequent study involved the comparison of time-lagged chronomodulated infusion profiles to better define the characteristics of optimal chronotherapeutic delivery (**Figure 9**). Two kinds of multiple-arm chronotherapeutic trials addressed the issue of tolerability as the main endpoint. In the first design, peak times of chronomodulated infusions are lagged over 24 h, yet with fixed intervals between the chronomodulated delivery patterns of the drugs in the combination. In 114 patients with metastatic colorectal cancer, peak times of oxaliplatin and 5-FU-leucovorin shifted by 3 h and compared with the reference profile, where delivery rate peaks at 4:00 p.m. for oxaliplatin and at 4:00 a.m. for 5-FU-leucovorin. This design assumed that it was important to maintain a fixed 12-h interval between the peak delivery rates of oxaliplatin and 5-FU-leucovorin (147). Severe toxicity occurred in 16.7% of the patients on the reference chronoFLO4 schedule and in 80% of those on the opposite chronomodulated modality (**Table 3**) (147). The optimal time of peak delivery rate was defined with its 90% confidence limits at 3:57 a.m. (11:30 p.m. to 9:36 a.m.) for 5-FU-leucovorin and at 3:57 p.m. (11:30 a.m. to 9:36 p.m.) for oxaliplatin. Such chronotolerance was confirmed for carboplatin and 5-FU-leucovorin in a randomized trial involving 45 patients with advanced non-small-cell lung cancer, receiving the three-drug chronomodulated schedules with peak drug delivery rates shifted by 8 hours (147). Patients treated with the reference profile experienced less frequent severe toxicity (6.7% versus up to 40%) and less frequent treatment delays or dose reductions (**Table 3**) (147).

Another design to find optimal times of administration involves the staggering of peak times of chronomodulated delivery of the single drug of interest every 3 or 4 h over 24 h (181). The other drugs in the combination are administered according to a fixed chronomodulated schedule. This results in varying intervals between the phases of the drug delivery profiles. In 90 patients with metastatic breast cancer, the peak delivery time of vinorelbine was shifted by 3, 6, 9, 12, 15, 18, or 21 hours, whereas peak delivery time of chronomodulated 5-FU was fixed at 4:00 a.m. (181). Estimated least leukopenia corresponds to peak vinorelbine delivery at 5.15 p.m. (2:12 p.m. to 8:08 p.m.), in good agreement with chronotolerance in female mice (182). Fewer dose reductions and/or treatment delays occurred for peak vinorelbine delivery at 8:13 p.m. (6:07 p.m. to 10:39 p.m.) (**Table 3**). Late evening vinorelbine tended to minimize the occurrence of severe neutropenia, febrile neutropenia, and gastrointestinal toxicities only at the higher dose tested (181). Both trial designs assume that the CTS and the clock-controlled pharmacologic pathways remain stable after being challenged by the first medication studied. However, vinorelbine can induce circadian disruption in mice (102). Similar designs can also help identify the optimal infusion duration, flow rate amplitude, and number of treatment days per course. The incorporation of translational endpoints is essential for the identification of patient subgroups and their corresponding distinct optimal chronomodulated schedules.

Relevance of chronomodulated delivery for efficacy. The relevance of a validated chronomodulated delivery regimen for antitumor efficacy was investigated in patients with metastatic colorectal cancer using tumor response rate and survival as the main criteria. Two consecutive European randomized trials compared chronoFLO5 with constant-rate infusion over 5 days every 3 weeks in a total of 278 patients. The percentage of patients whose metastases regressed by $\geq$50% was 29% on constant-rate infusion and 51% on chronomodulated delivery ($p < 0.001$). However, overall survival did not significantly differ according to treatment schedule (17, 148, 149). A third randomized trial compared the chronomodulated administration of the same three drugs over 4 days (chronoFLO4) with a 2-day conventional administration schedule without any timing stipulation (FOLFOX2) in 564 previously untreated patients with metastatic colorectal cancer (183). The trial was intended to treat each patient at the near maximum tolerated dose. Overall survival, the main endpoint in this large international study, did not differ as a function of treatment schedule. However, the relative risk of an earlier death on chronoFLO4 significantly increased by 38% in women and significantly decreased by 25% in men compared with conventional delivery (183). A recent meta-analysis of these three randomized trials in 842 patients with metastatic colorectal cancer confirms that the three-drug chronomodulated infusion achieves similar or worse efficacy compared with conventional delivery in women. In men, however, the same chronoFLO treatment significantly increases tumor response and survival compared with conventional delivery, independent of other prognostic factors (**Table 3**) (184).

Three hypotheses are currently being tested to account for such gender-schedule interactions: (*a*) the occurrence of a different circadian genotypic profile between males and females with colorectal cancer, (*b*) the sex dependency of circadian pharmacology of anticancer drugs, and (*c*) the occurrence of excessive toxicity in women causing circadian disruption, and thus the impairment of chronotherapeutic mechanisms. This hypothesis is supported by the occurrence of 20% to 50% more toxicities in women (147, 183). Preliminary studies show that cancer treatments can disrupt the rest-activity rhythms in cancer patients, and this disruption is associated with systemic toxicities (185).

TOWARD THE PERSONALIZATION OF CANCER CHRONOTHERAPEUTICS

The standardization of cancer chronotherapeutics has been mostly developed using male B6D2F1 mice and successfully transferred to the clinic. However, clinical and translational data also show differences in circadian rhythms of individual cancer patients that are relevant for therapeutic outcomes. Dedicated in vitro, in vivo, and in silico experimental models and technologies are paving the way to personalized cancer chronotherapeutics.

Circadian Physiology and Clock Genes in Cancer Patients

Circadian alteration or disruption of plasma cortisol and melatonin, blood cell count, liver enzymes, or renal tests were observed in individual patients (176, 186). The patients with near normal cortisol rhythm had synchronous circadian rhythms in bone marrow S-phase cells and 5-FU concentrations during constant-rate infusion for 24 h. In contrast, the patients with damped or ablated cortisol rhythm displayed blunted, if any, rhythmic patterns in bone marrow or plasma 5-FU concentrations (170, 187). Minimally invasive techniques, such as rest-activity monitoring or iterative salivary cortisol determinations, provided CTS estimates in large populations of cancer patients that match those routinely treated for cancer (12, 13). Nearly a third of patients with metastatic breast or colorectal cancer displayed poor rhythms in salivary cortisol and/or rest-activity before

they received chemotherapy (11–13). Moreover, circadian disruption appears as an independent prognostic factor of survival (11–13) and hinders both chronotherapeutic mechanisms and host control of malignant processes.

Human clock genes are highly polymorphic, as documented by large population-based studies (188, 189). The phenotype associated with germline variants of clock genes can affect not only sleep preferences, mood disorders, and metabolic diseases but also cancer risk. Extreme circadian rhythm–related sleep disorders, such as familial advanced or delayed sleep syndromes are caused by polymorphisms in *Per2* and *Csnk1d*, and *Clock* and *Per3* genes, respectively (189, 190). Less extreme chronotypes of morningness or eveningness preferences seem to be also more frequently associated with genetic variants of *Per2*, *Per3*, *Clock*, and *Csnk2a2* (189, 190). Polymorphisms of the clock gene *Npas2*, a *Clock* homolog that predominates in specific tissues, are associated with a decreased risk of non-Hodgkin's lymphoma and breast and prostate cancers (191–193). Conversely, *Cry2* polymorphisms are associated with an increased risk of non-Hodgkin's lymphoma and prostate cancer (191, 194). A limited study in patients with esophageal cancer found no significant association between 5-FU circadian pharmacokinetics and a *Clock* gene polymorphism, reported to affect the time course of antidepressant-induced insomnia (195, 196). Polymorphisms in tissue-specific clock-controlled genes can also account for interindividual differences in relevant circadian rhythms, as shown for plasminogen activator inhibitor type I (PAI-1) that circulates rhythmically with a morning peak in human peripheral blood. Genomic variants within the gene promoter region critically determine 24-h changes of plasma concentrations of PAI-1, from severely blunted to severalfold (197). Similarly, rhythmic patterns in locomotor activity and temperature rhythms are affected by polymorphisms in a serotonin reuptake transporter in depressive patients (198, 199).

In human tumors, the mRNA or protein expression of the clock genes *Per1*, *Per2*, or *Per3* as well as *Npas2* or *Dec1* is markedly decreased on average or deregulated in comparison with reference tissues. This is the case for cancers of the breast, lung, colon, endometrium, ovary, pancreas, and bone marrow, a finding supporting frequent circadian disruption in human malignancies (32, 34, 152, 200). The altered expression of clock genes in human tumors can influence the efficacy of cancer chronotherapeutics. The mRNA expression of *Per1* and *Dpyd* are strongly correlated in primary human colorectal cancers, and more so in women. The relation between *Per1* and *Dpyd* expression is disrupted in poorly differentiated colon carcinoma cells, possibly resulting in the suppression of tumor *Dpyd* oscillations (200).

Chronopharmacology at the Cellular Level

The circadian transcriptome has been determined using microchip DNA arrays on iterative samples from synchronized cultures of mouse or rat fibroblast or immortalized SCN cell lines (201, 202). Rhythmic mRNA expression is demonstrated in cell cultures for genes encoding for transcription factors, such as *Dbp*, cell cycle, apoptosis, differentiation, glucose metabolism, and detoxification, with tissue specificity (201, 202). Synchronized cell cultures display rhythmic transcription of drug metabolism and targets, such as *Cyp2e1*, *Cyp3a4*, and *Top1* (90, 203, 204). Ex vivo cell cultures of bone marrow progenitors from male B6D2F1 or Balb/C mice show sustained circadian rhythms in pharmacodynamic response to granulo-monocytic colony-stimulating factor (GM-CSF) over 4 days (205). The circadian maximum in the proliferative response of bone marrow cells to GM-CSF occurs at the same circadian time in vitro and in vivo (205). Ex vivo bone marrow liquid cell cultures are also synchronized with serum shock and thus can be used for investigating cellular chronopharmacology in hematopoietic cells (206). The circadian period and amplitude of luminescent-reporter human osteosarcoma cells are novel clock-related endpoints that can be used to screen for pharmacologically active compounds (207). The lengthening of

the circadian period has been shown to be a novel pharmacologic property of seliciclib, a finding confirmed by our team in mice with a free running CTS. The colon adenocarcinoma cells Caco-2 express clock genes and proteins in synchronized cell cultures (158). Caco-2 cells are also used as an in vitro model to investigate pharmacokinetic-pharmacodynamic (PK-PD) relations of anticancer drugs (158, 208, 209). Synchronized undifferentiated Caco-2 cells display coordinated circadian transcription patterns of the clock genes *Rev-erbα*, *Per2*, and *Bmal1* and the irinotecan pharmacology genes *Ces2*, *Ugt1a1*, *Abcb1*, *Abcc1*, *Abcc2*, *Abcg2*, and *Top1* (**Figure 10**) (210). Such an in vitro system allows the dynamic determination of chronoPK-PD relations for building

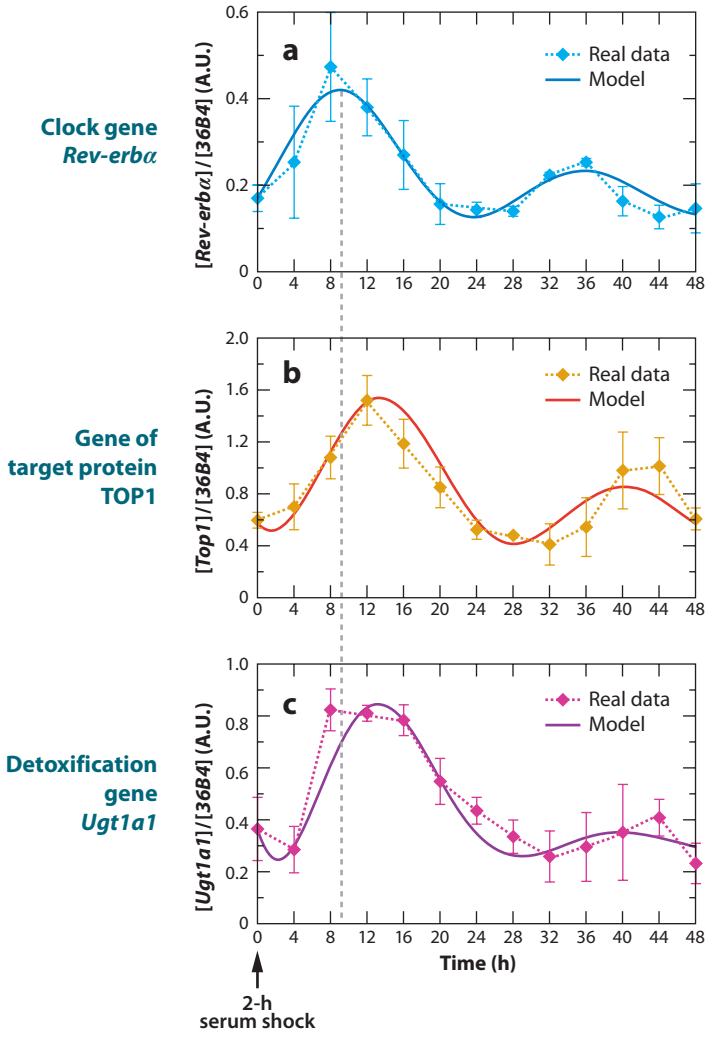


Figure 10

Synchronized in vitro model of cellular determinants of irinotecan chronopharmacology. Temporal changes of mRNA expression of (*a*) *Rev-Erbα*, (*b*) *Top1*, and (*c*) *Ugt1a1* in undifferentiated Caco-2 cells over the 48 h following synchronization of cell cultures with 2-h serum shock (*vertical arrow*). Actual data have been normalized to housekeeping gene *36B4* (*dotted lines*) and output functions from mathematical model incorporating circadian clocks and decay functions (*solid lines*).

first-generation chronotherapeutic models at the cellular level, with the perspective of personalizing cancer chronotherapeutics.

The properties of individual human circadian clocks can be determined using ex vivo fibroblasts from skin biopsies following transformation with lentiviral *Bmal1-luciferase* reporter (211). The continuous monitoring of *Bmal1* transcription with luminescence detectors reveals that the circadian period of *Bmal1* differs from 24.3 ± 0.4 h in the subjects with an early chronotype to 24.7 ± 0.3 h in those with a late chronotype (211).

Chronotoxicity Classes in Mouse Models

The pharmacologic effects of anticancer drugs differ largely according to cell lines and species, strain, or gender of experimental animal models. The heterogeneity of cancer cells and cancer tissues is an additional cause of variability of anticancer drug effects. For instance, strain-specific differences in glucuronidation reactions and irinotecan detoxification characterize C57/Bl6, DBA2J, and BALB/c mice and result in strain-dependent toxicity and efficacy of this drug (81). The overall tolerability of theprubicin in B6D2F1 mice is approximately threefold better in females than in males. Moreover, the optimal circadian timing for tolerability occurs at ZT10 in males and at ZT14 in females. The magnitude of chronotolerance for the highest dose tested is 8-fold in male and 0.6-fold in female B6D2F1 mice (F. Lévi, unpublished data). In contrast, the optimal circadian timing of theprubicin is located at ZT15 in male C57/Bl6 mice (46). Optimal circadian timing improves cisplatin tolerability threefold at ZT19 in female F344 Fischer rats and twofold at ZT15 in male B6D2F1 mice compared with dosing 12 h apart (59, 212). Not only the average level of irinotecan tolerability but also its circadian pattern differ significantly as a function of mouse strain and sex. Optimal circadian timing of irinotecan is at ZT11 in male and at ZT15 in female B6D2F1 mice. The highest plasma AUC of CPT-11 and SN-38 results from irinotecan administration at the most toxic time in female B6D2F1 mice (82). However, no such consistent relationship between circadian pharmacokinetics and toxicity is found in male mice of the same strain (82). The identification of underlying molecular and physiological circadian determinants of distinct chronotoxicity classes may subsequently guide the clinical development of tailored cancer chronotherapeutics.

NOVEL INSIGHTS INTO CANCER CHRONOTHERAPEUTICS THROUGH MATHEMATICAL MODELS

The Optimization of Chronotherapeutic Delivery under Chronotoxicity Constraints

Mathematical models of cancer chronotherapeutics are designed with the goal of therapeutic optimization. Just as cell population dynamics models are used to represent tumor and healthy tissue kinetics, optimization consists of finding the best possible infusion time schedule (solution to an optimal therapeutic control problem) that can be used to minimize a population of cancer cells: its absolute minimum, or in contrast its maximum within a treatment window, if one only wants to stabilize rather than eradicate the tumor. This minimum or maximum is the objective function to be minimized by therapeutic control. Optimization is always performed under constraints. The main constraint by far is to respect host tolerance for anticancer medications. This requires the definition and the quantification of the healthy cell populations to be shielded from toxic insult, using parameters that depend both on the drug and on the tissues turning over quickly, such as bone marrow, gut, and skin. Other constraints such as maximum total dose and maximal drug

infusion flow are easily defined and are usually the only ones that are considered in conventional chemotherapeutics, yet they are inappropriate for cancer chronotherapeutics.

For such a treatment strategy, one needs to consider toxicities in a dynamic way, by defining infusion schedules that vary along the circadian time span. A toxic threshold for the healthy target tissue is physiologically defined, not as a drug concentration or dose, but as a lower-limit cell population number under which it would be hazardous (potentially lethal) to descend. Such a limit parameter drives the adaptation of the drug infusion flow rate in individual patients. To accurately define such a limit and adapt it to the delivered drug infusion flow, cell population dynamics models are designed with a physiological structure, that is, with respect to cell cycle progression, cyclin concentrations, etc., including control targets, to allow the representation of the mechanisms of drug toxicity (213).

This formulation of the chronotherapeutic optimization problem in terms of objective and constraint functions allows the use of mathematical methods such as optimal control on a physiological, not only an empirical, basis. This is illustrated in a proof of principle study of in silico chronotherapeutics with oxaliplatin. Parameter identification is performed on tumor growth curves in mice, with a simplified PK-PD model based on the jejunal toxicity and the antitumor efficacy of oxaliplatin (61, 66, 80, 177, 180). The solution to the optimal control problem is a theoretically optimized drug infusion flow (**Figure 11**). The treatment constraints critically determine the optimal chronotherapeutic schedule. Interestingly, constant rate infusions always achieve worse therapeutic outcomes than optimized chronomodulated regimens in these models. The same principles are currently driving the in silico optimization of chronomodulated drug delivery schedules to be administered with multichannel programmable pumps in cancer patients.

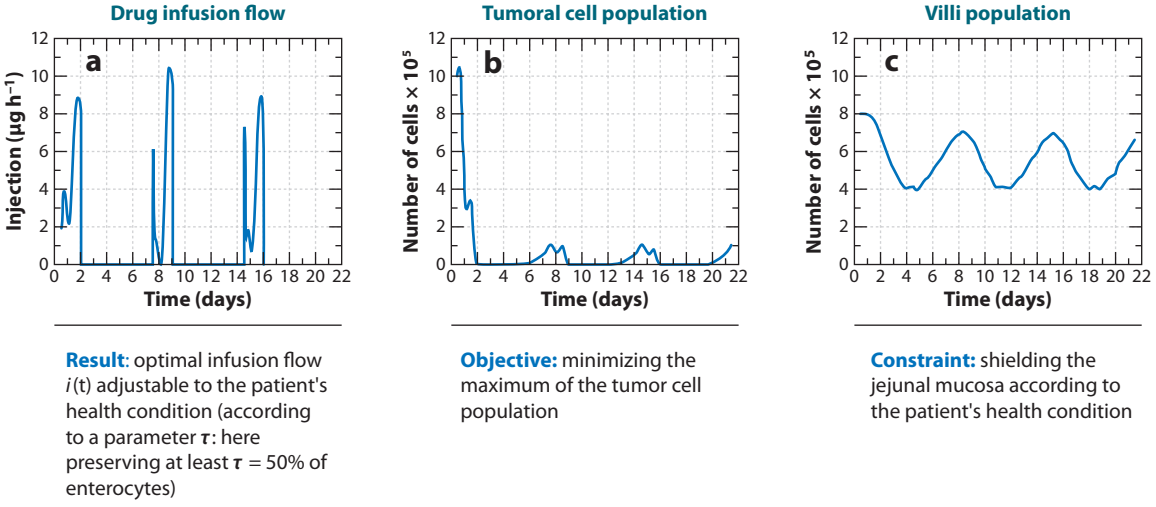


Figure 11

Relevance of optimal control theory for designing chronotherapeutic delivery schedules. Results of an optimal control problem using a pharmacokinetic-pharmacodynamic model of single agent oxaliplatin for parameter identification in tumor-bearing mice. (*a*) Numerical solution to the optimal control problem resulting in an optimized chronomodulated drug delivery schedule. (*b*) Dynamics of the tumor cell population under the objective function of minimization of its local maxima. (*c*) Dynamics of the healthy cell population under the constraint of keeping it above a given threshold. Adapted from Reference 234.

Relation Between Chronotoxicity and Chronoefficacy

Chronotoxicity and chronoefficacy involve similar molecular mechanisms at the cell level, and thus can be represented in the same way by the action of drugs on molecular cell targets. However, anticancer drugs can elicit different responses in healthy or malignant cell populations. Physiologically based PK-PD mathematical models help us understand the molecular mechanisms that discriminate the differential circadian response of healthy cells and cancer cells to anticancer drugs. Thus, in silico testing can probe the respective relevance of determinants of cell cycle control, apoptosis, and DNA repair in cell population dynamics, including the role of p53 mutational status, but they can also be found in drug-processing cell mechanisms, including reduced glutathione and its synthesis, and specific enzymes, such as DPYD or UGT1A1. Another important discriminating property of cancer cells is their ability to express ABC transporters, including ABCB1, that transport xenobiotics from inside the cell to the outside. Many of the mechanisms mentioned above show circadian modulation of their activity, at least in normal cells, so that poor or absent sensitivity to CTS inputs in cancer cells could account for observed differences of responses to time-scheduled drug regimens with respect to chronoefficacy and chronotolerance.

The differences in drug response with respect to host and tumor circadian clocks, which are intended to be exploited by optimized chronotherapeutic schedules, may thus be due to the disruption of mechanisms at the individual cell level, but they may also be due to disruption of the physiological synchrony with respect to phases of the cell division cycle in normal proliferating cell populations (e.g., gut, bone marrow). From observations on circadian clock–controlled gene expression in tumors and in healthy tissues, it has been hypothesized that (*a*) the CTS is an essential coordinator, and (*b*) poor circadian synchronization offers a proliferation advantage for cancer cell populations over well-synchronized healthy cell populations. Although no causal relationship between poor synchronization and enhanced proliferation has been established experimentally, the desynchronization between tumor cells and healthy cells provides possible insights into the relation between chronoefficacy and chronotoxicity (177, 179, 214). The use of a cellular automaton model reveals that strong circadian synchronization among cells is an essential feature for healthy cell populations in order to display a phase of low susceptibility to cytostatics. In contrast, cancer cells, with blunted synchronization, remain sensitive to the action of the drug with low variation in susceptibility. This alone might explain the differential response of healthy and malignant cell populations to optimally tolerated chronomodulated chemotherapy (214). The model further reveals that the cell cycle duration, which can differ between tumor and healthy cell populations, but also among individual tumor cells, is another critical parameter (214). Taking into consideration both cell synchronization with respect to cell cycle phases and cell cycle duration, theoretically optimized infusion schemes, which should achieve the best tumor containment and best preservation of healthy cells, can be generated.

The improved knowledge regarding time determinants in the physiological mechanisms at work (drug detoxification, cell cycle control, cell population synchrony) is bringing more flexibility to the dynamic delivery of cancer treatments to individual patients. The shielding of the patient's CTS from treatment-induced disruption represents a new dynamic constraint that could be prevented through adequate drug delivery patterns, early detection through dedicated technology, and rapid correction through feedback into chronotherapeutic algorithms to further optimize chronotherapeutic patterns along the course of cancer treatments in individual patients.

Mathematical Models and Technologies for Tailoring Chronotherapeutic Delivery

Mechanistic models can include any relevant intracellular mechanism involved such as polymorphisms of genes and the dynamic organization and treatment responses of cells, tissues, and whole organisms. For example, genetic differences in 5-FU catabolism by DPYD can be taken into account by different Km and Vmax values of the enzyme activity (**Figure 12**). Physiologically based mathematical models designed to propose theoretically optimized chronotherapeutic delivery schedules must consist of (215):

1. Dynamic models of cell populations, physiologically structured, with structure variables describing evolution in the cell-division cycle for both healthy and cancer cell populations (with different parameters) and with prescribed targets (parameters of the population dynamics model) for circadian and pharmacological control (213).
2. Molecular chronoPK-PD models for anticancer drugs, including action exerted on their targets in cell population models (pharmacodynamics), intracellular transformation of drugs by enzymes, active efflux proteins or other intracellular agents (intracellular pharmacokinetics), and drug fate from its infusion in the general circulation, possibly via previous intestinal absorption, and certainly with hepatic detoxification, until its delivery to peripheral cells, thus calling for the design of a whole-body compartmental chronoPK model.
3. Optimal control methods of chronomodulated drug infusion rates dealing with several drugs administered simultaneously (180). These methods handle several targets on cell population dynamics, one objective function to be minimized (i.e., a measure of the tumor cell population), and constraints that involve (*a*) at least preservation of an absolute number of the healthy cell population that is the target of toxicity, (*b*) prevention of the occurrence of a resistant tumor cell clone, and (*c*) preservation of functional circadian clocks.

CONCLUSION AND PERSPECTIVES

Through its multilevel hierarchical organization, the CTS controls the metabolism, detoxification, and pharmacodynamic effects of anticancer drugs of all pharmacologic classes, both in experimental models and in cancer patients. Common features characterize the CTS and clock-controlled pathways relevant for cancer chronotherapeutics in living beings. Monitoring of circadian rhythms and programmable-in-time drug delivery technologies enable the translation of the

Figure 12

Mathematical model combining intracellular molecular chronopharmacokinetics and chronopharmacodynamics of 5-FU and leucovorin (LV) on the intracellular target enzymethymidilate synthase (TS). (*a*) In this system of ordinary differential equations, where dynamic variables are concentrations, drug inputs *i* and *j* are the plasma infusion flows of 5-FU and LV, respectively, and the pharmacodynamic output is the blockade of TS under the form of a stable ternary complex, secondarily degraded, but which irreversibly consumes free TS when it is formed. The intracellular active compounds, FdUMP for 5-FU and methylene tetrahydrofolate (MTHF) for LV, exert their action on TS by yielding first a reversible binary complex B binding 5-FU and TS, and then the irreversible ternary complex T by the adjunction of MTHF (pharmacodynamic part: Equations 7, 8, 9). The intracellular pharmacokinetic part of the model, in addition to simple transport and intracellular transformation for LV (Equations 3 and 4), describes (Equation 1) the degradation of 5-FU (variable *P*) in the liver, considered as a filter inside the plasma compartment, by hepatic dihydropyrimidine dehydrogenase (DPYD) and, through a saturable mechanism, its entry in the cell, where (Equation 2), under the form of FdUMP (variable *F*), either it is expelled by an FdUMP-triggered—via a nuclear factor (variable *N*)—ABC transporter (variable *A*), as represented in Equations 5 and 6, or it binds to free TS (variable *S*). Hepatic DPYD and intracellular TS are represented with their circadian rhythms by a cosine-like modulation. (*b*) The physiological basis of the variables considered is illustrated by a symbolic representation of the plasma compartment (tubular) where drug inputs i and j are infused and that of the intracellular compartment (*ellipse*) where biochemical reactions occur, involving physiological and pharmacological compounds.

a

$$\textbf{1}\quad \frac{dP}{dt} = -k_0 P - \frac{aP}{b+P} - l_{DPYD}\,\frac{P}{m_{DPYD}+P} + \frac{i(t)}{V}$$ ← Input i = 5-FU infusion flow

$$\textbf{2}\quad \frac{dF}{dt} = \frac{a}{\xi}\,\frac{P}{b+P} - \frac{AF}{c+F} - k_1 FS + k_{-1} B$$

$$\textbf{3}\quad \frac{dQ}{dt} = -k_2 Q + \frac{j(t)}{V}$$ ← Input j = LV infusion flow

$$\textbf{4}\quad \frac{dL}{dt} = \frac{k_2}{\xi} Q - k_3 L - k_4 BL$$

$$\textbf{5}\quad \frac{dN}{dt} = \frac{\varkappa F^n}{\lambda^n + F^n} - \mu N$$

$$\textbf{6}\quad \frac{dA}{dt} = \mu N - \nu A$$ ← A = ABC transporter (active drug efflux)

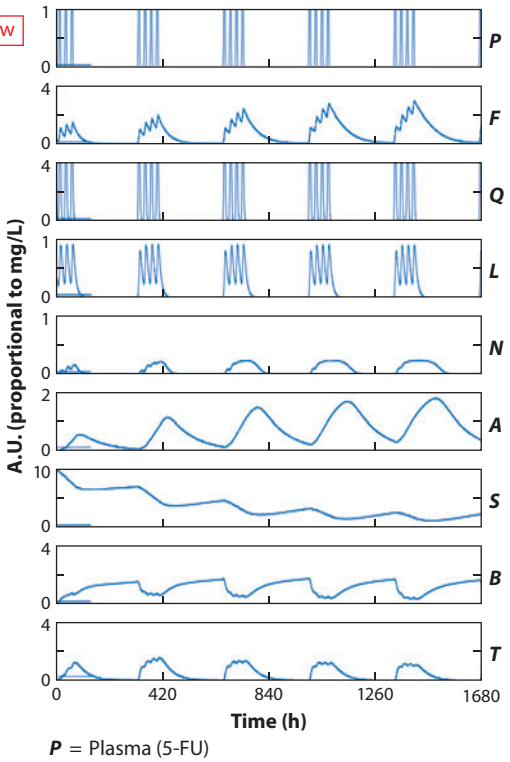
$$\textbf{7}\quad \frac{dS}{dt} = -k_1 FS + k_{-1} B + \theta_{TS}\,(S_0 - S)$$ ← S = free thymidylate synthase (TS)

$$\textbf{8}\quad \frac{dB}{dt} = k_1 FS - k_{-1} B - k_4 BL$$

$$\textbf{9}\quad \frac{dT}{dt} = k_4 BL - \nu_T T$$ ← Drug output T = blocked thymidylate synthase (stable ternary FdUMP-MTHF-TS complex)

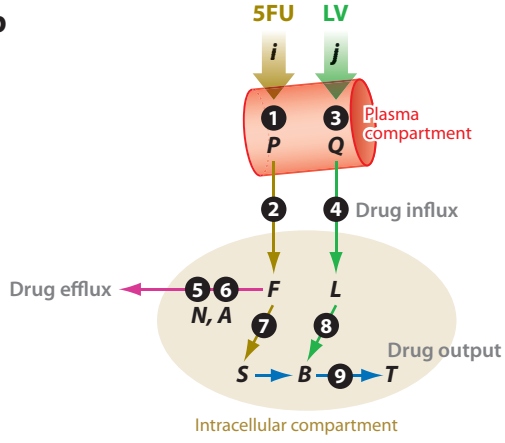
where $l_{DPYD} = l_{DPYD\_BASE}\left\{1 + \varepsilon \cos\frac{2\pi(t - \phi_{DPYD})}{24}\right\}$

and $S_0 = S_{0\_BASE}\left\{1 + \delta \cos\frac{2\pi(t - \phi_{TS})}{24}\right\}$



P = Plasma (5-FU)
F = Intracellular (FdUMP)
Q = Plasma (LV)
L = Intracellular (MTHF)
N = 5-FU-triggered nuclear factor
A = ABC transporter activity, nuclear factor-induced
S = Free (TS) (not FdUMP-bound)
B = (FdUMP-TS) reversible binary complex
T = (FdUMP-TS-LV) stable ternary complex

b



severalfold improvement of the tolerability and efficacy of cancer treatments from experimental models to cancer patients. Standardized chronotherapeutics involves the administration of anticancer drugs with fixed chronomodulated profiles and fixed circadian timing for all the patients. Adequate chronomodulated schedules usually reduce interpatient variability in drug pharmacokinetics compared with conventional administration of drugs. The clinical development of standardized chronotherapeutics was the first to reveal the safety and antitumor activity of oxaliplatin in patients with metastatic colorectal cancer, to trigger a new medical and surgical strategy with curative intent for this disease, and to specify the need for chronomodulated infusion protocols for nonhospitalized patients, with persistent circadian synchronization. However, CTS components can be variably altered among cancer patients, despite consistent group patterns in most circadian biomarkers. These observations call for the definition of subgroups of patients with distinct circadian characteristics.

After decades of therapeutic development ruled by the standard dosing regimens, the personalization of treatment becomes a central goal in the field of cancer. However, the tools and methods to achieve such a goal remain elusive. Database mining and translational circadian studies of cancer patients, involving physiological, pharmacogenetic, and pharmacogenomic endpoints are required (**Figure 13**). These translational investigations need to be guided by new dedicated experimental models. Thus, the circadian map of the molecular mechanisms of anticancer drug

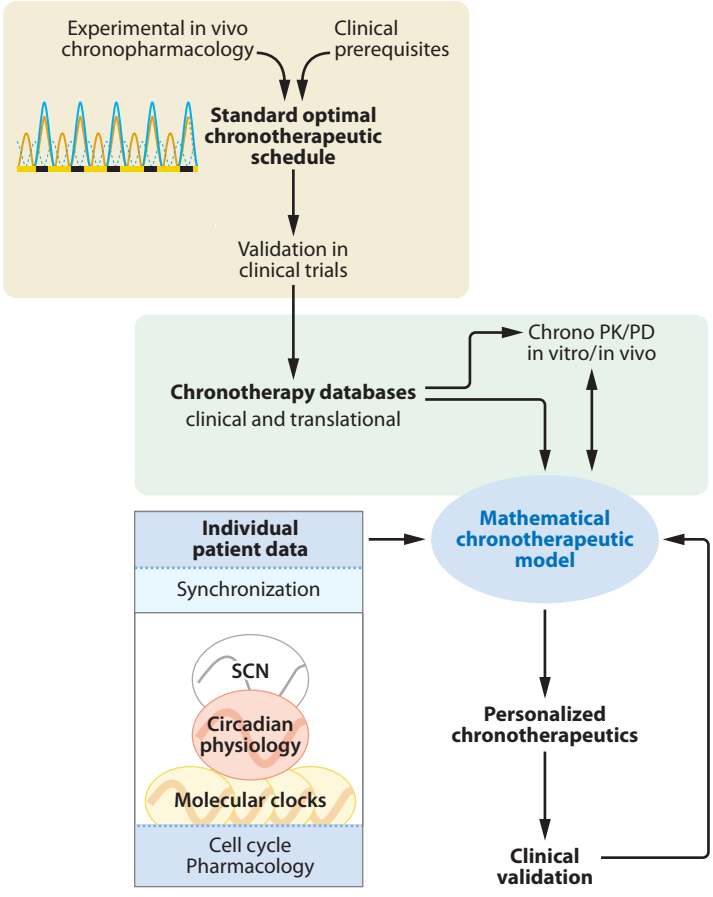


Figure 13

Main steps in developmental chronotherapeutics of cancer, from standardization to personalization.

chronopharmacology currently helps in building theoretical chronotherapeutic models at cellular, tissue, and whole organism levels. Chronopharmacokinetic and chronopharmacodynamic data from synchronized cell cultures and from mice of different genders and strains are being integrated to design distinct optimal chronotherapeutic patterns for anticancer drug delivery according to CTS status and dynamics (**http://www.biosim-network.net/** and **http://www.chronotempo.org/**). The paradigm of personalized chronotherapeutics for cancer is stimulating the development of novel cancer treatment algorithms both for chronomodulated drug delivery and for the selective targeting of defective circadian clocks or clock-controlled pathways.

Technological advances now allow for complex drug delivery after both systemic and oral administration routes through programmable pumps and oral multiple-unit preparations (216–218). Novel drug delivery systems could enable the personalization of chronotherapeutics with oral anticancer drugs through patient- and drug-specific preparations, thus contributing to improvement of the currently limited tolerability and efficacy of these agents.

The acquisition of relevant temporal information in individual patients through dedicated technologies will enable a systems chronopharmacology approach to optimally adjust the dynamic patterns of anticancer drug exposure to the circadian timing system of the individual cancer patient.

SUMMARY POINTS

1. The endogenous circadian timing system rhythmically controls cellular metabolism and proliferation, which determine the pharmacologic effects of anticancer agents.
2. Circadian timing significantly modifies tolerability and efficacy in experimental models and in cancer patients.
3. Mechanisms involve the circadian control of phase I and II metabolism and that of cell cycle checkpoints and apoptosis.
4. Optimal circadian timing and dosing of anticancer drugs can differ according to gender.
5. Studies in male mice translate into large and significant improvements in tolerability and efficacy in male patients with cancer.
6. In vitro, in vivo, and in silico models of cancer chronopharmacology are leading toward the personalization of cancer chronotherapeutics.

FUTURE ISSUES

1. In vitro models of anticancer drug chronopharmacology need to be developed and diversified.
2. Mathematical models will integrate the reciprocal signaling between circadian clocks and drug metabolism, cell cycle, DNA repair, and apoptosis in healthy and cancer cells through systems biology approaches.
3. Multiple preclinical models with distinct clock properties are required for the personalization of cancer chronotherapeutics and the prediction of optimal chronomodulated drug delivery.
4. The stages where chronotherapeutics will be integrated into the development of new anticancer drugs will have to be defined, ranging from screening to clinical phases.

5. Dedicated diagnostic technologies are needed for dynamic quantitative and noninvasive assessment of circadian timing system components.
6. Multiple dedicated drug delivery technologies will enable the ambulatory administration of personalized cancer chronotherapeutics.

DISCLOSURE STATEMENT

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

ACKNOWLEDGMENTS

The authors acknowledge the support of their research by the European Union through the Network of Excellence BIOSIM (biosimulation: a new tool for drug development; contract LSHBCT-2004-005137), the Specific Targeted Research Project TEMPO (Temporal Genomics for Tailored Chronotherapeutics; contract LSHG-ct-2006-037543), the Specific Support Action PROUST (The Temporal Dimension in Functional Genomics; contract LSSG-CT-2006-037654), the Association Internationale pour la Recherche sur le Temps Biologique et la Chronothérapie (ARTBC International), and hospital Paul Brousse, Villejuif (France). A.O. is a recipient of a postdoctoral fellowship from the Scientific and Technological Research Council of Turkey (TUBITAK).

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Volume 50, 2010

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Errata

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