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Title Page

Circadian clock-controlled drug metabolism: Implications for chronotherapeutics

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Running Title Page

Running Title: Metabolism-based chronotherapeutics

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Non-standard abbreviations

AF, activation function; APAP, acetaminophen; Bcrp, breast cancer resistance protein; Bmal1, brain and muscle Arntl-like protein 1; Car, constitutive androstane receptor; Ces, carboxylesterase; Clock, circadian locomoter output cycles kaput; CPA, cyclophosphamide; Cry, crytochrome; Cyp, cytochrome P450; Dbp, albumin D site-binding protein; E4bp4, E4-binding protein 4; Fmo, flavin-containing monooxygenase; Hdac3, histone deacetylase 3; Hnf4α, hepatocyte nuclear factor 4 alpha; Lrh-1, liver receptor homolog 1; Lxr, liver X receptors; Mrp, multidrug resistance-associated protein; Ncor1, nuclear receptor corepressors 1; Npas2, neuronal PAS domain protein 2; NR, nuclear receptor; Per, period; P-gp, P-glycoprotein; Ppar, peroxisome proliferator activated receptor; Pxr, pregnane X receptor; SCN, suprachiasmatic nucleus; RevRE, Rev-erb response element; Ror, RAR related orphan receptor; RORE, Ror response element; Rxr, retinoid-X receptor; Shp, small heterodimer partner; Sult, sulfotransferase; Ugt, UDP-glucuronosyltransferase; Vdr, vitamin D receptor; ZT, zeitgeber time.

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Abstract

Dependence of drug metabolism on dosing time has been long recognized. However, it is until recent years that the underlying mechanisms for circadian drug metabolism are being clarified. Diurnal rhythmicity in expression of drug-metabolizing enzymes is believed to be a key factor determining circadian metabolism. Supporting the notion that biological rhythms are generated and maintained by the circadian clock, a number of diurnal enzymes are under the control of circadian clock. In general, circadian clock genes generate and regulate diurnal rhythmicity in drug-metabolizing enzymes via transcriptional actions on one or two of three *cis*-elements (i.e., E-box, D-box, and RevRE or RORE). Additionally, cycling or clock-controlled nuclear receptors such as Hnf4α and Ppar-γ are contributors to diurnal enzyme expression. These newly discovered mechanisms for each of rhythmic enzymes are reviewed in this article. We also discuss how the rhythms of enzymes are translated to circadian pharmacokinetics and drug chronotoxicity that has direct implications for chronotherapeutics. Our discussion is also extended to two diurnal transporters (P-gp and Mrp2) that have an important role in drug absorption. Although the experimental evidence is lacking in metabolism-based chronoefficacy, circadian genes (e.g., *Rev-erbα*) as drug targets are shown to account for diurnal variability in drug efficacy.

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Significance Statement

Significant progresses have been made in understanding the molecular mechanisms for generation of diurnally rhythmicity in drug-metabolizing enzymes. In this article, we review the newly discovered mechanisms for each of rhythmic enzymes, and discuss how the rhythms of enzymes are translated to circadian pharmacokinetics and drug chronotoxicity that has direct implications for chronotherapeutics.

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Introduction

It has been long recognized that the effects of many drugs depend on dosing time (time of administration) with a variability of up to 10-fold (Lévi and Schibler, 2007; Dallmann et al., 2014). To date, time-varying effects have been documented for over 300 medications (Baraldo et al., 2008; Bruguerolle, 1998; Innominato et al., 2010; Lévi et al., 2011; Kaur et al., 2013; Ohdo et al., 2019; Ruben et al., 2019). Strikingly, chronotherapy with drugs generates better efficacy (about 2-fold) and tolerability (up to 5-fold) compared with conventional therapy (Iurisci et al., 2009; Koyanagi et al., 2003; Lévi et al., 2003, 2011). The mechanisms for time-dependent drug effects appear to be complicated. Of note, circadian pharmacokinetics (or called chronopharmacokinetics) may be one of main sources of time-varying drug effects (Baraldo et al., 2008; Ruben et al., 2019). Dependence of pharmacokinetics on dosing time has been described for over 50 drugs in humans (Dallmann et al., 2014; Ohdo et al., 2019). Unfortunately, the molecular mechanisms underlying these chronopharmacokinetic events remain largely unknown.

Metabolism (biotransformation catalyzed by drug-metabolizing enzymes) is a main defense mechanism of the body against xenobiotic threats, and regarded as a key determinant of pharmacokinetics (and drug exposure) and therefore of pharmacological effects (Benedetti et al., 2009; Wilkinson, 2005). On the other hand, toxic metabolites may be generated from metabolism reactions, causing adverse effects and disfavoring new drug development (Guengerich, 2006). Over 50 years ago, Radzialowski & Bousquent (1968) reported dosing time-dependent drug metabolism

in rodents, suggesting a potential role of circadian metabolism in determining chronopharmacokinetics. From then on, great progresses have been made in understanding the molecular mechanisms underlying rhythmic expression of drug-metabolizing enzymes. These newly discovered mechanisms for each of rhythmic enzymes are reviewed in this article. We also discuss how the rhythms of enzymes are translated to circadian pharmacokinetics and drug chronotoxicity that has direct implications for chronotherapeutics. Our discussion is also extended to two diurnal transporters (P-gp and Mrp2) that have an important role in drug absorption.

Drug-eliminating system

The body possesses a sophisticated system to eliminate drugs. Historically, drug elimination consists of phase I metabolism, phase II metabolism, and phase III excretion (Almazroo et al., 2017; Döring and Petzinger, 2014). Phase I metabolism (modification reactions) include oxidation, reduction, and hydrolysis that introduce new functional groups such as hydroxyl, carboxyl, and amino groups into the drug structure (Testa et al., 2012). Enzymes involved in phase I reactions include CYPs (cytochromes P450), FMOs (flavin-containing monooxygenases), MAOs (monoamine oxidases), AOXs (aldehyde oxidases), ADH (alcohol dehydrogenase), ALDH (aldehyde dehydrogenase), and CESs (carboxylesterases). In phase II reactions, drugs are conjugated with a hydrophilic group, generating polar metabolites that are more excretable (Testa et al., 2012). Major phase II enzymes are the UDP-glucuronosyltransferases (UGTs), sulfotransferases (SULTs), glutathione S-transferases (GSTs), and arylamine N-acetyltransferases (NATs). CYPs are major role

players in phase I metabolism of drugs/xenobiotics and endogenous compounds such as steroid hormones (Zanger and Schwab, 2013). UGTs-mediated glucuronidation reactions account for a high portion (~35%) of phase II drug metabolism (Meech et al., 2019). Overall, CYPs and UGTs contribute to 40% and 14% of total drug metabolism, respectively (Testa et al., 2012). Of 125 FDA-approved drugs (2006-2015), formation of major metabolites (≥10% of drug dose) is primarily catalyzed by CYPs (52.5%), followed by UGTs (11.7%) (Cerny, 2016).

Efflux transporters or exporters are a class of transporters that mediate the phase III excretion process. P-gp (P-glycoprotein), MRPs (multidrug resistance-associated proteins) and BCRP (breast cancer resistance protein) are the main transporters in efflux transport of drugs and metabolites (Xu et al., 2005; Döring and Petzinger, 2014). Transporter-mediated excretion is necessary for many hydrophilic drug molecules and metabolites (particularly phase II metabolites) because they cannot passively diffuse out of cells (Schinkel and Jonker, 2003; Choi and Yu, 2014). The liver, intestine and kidney (known as major drug-eliminating organs) express high levels of phase I and II enzymes as well as efflux transporters (Schaefer et al., 2012; Nakamura et al., 2016; Ohtsuki et al., 2012).

The crosstalk between drug metabolism and transport has been long recognized, a phenomenon termed “enzyme-transporter interplay” (Benet, 2009). The most famous example is the CYP3A-P-gp interplay. Such interplay has implications for better understanding of pharmacokinetics and bioavailability of drugs that are substrates of both CYP3A and P-gp (Christians et al., 2005). Mechanistically, drug substrates have

more chance to encounter the enzymes (CYP3A) due to P-gp-mediated excretion and reabsorption, resulting in enhanced drug metabolism and clearance (Mudra et al., 2011). In addition, the interplay between phase II enzymes and efflux transporters has been also well characterized (Jeong et al., 2005; Wu, 2012; Wang et al., 2016). Chemical inhibition or genetic knockdown of Mrps/Bcrp leads to reduced conjugation of drug/xenobiotic, highlighting dependence of cellular metabolism on efflux transport.

Circadian clock system

The rotation of the Earth causes daily changes in the environment, such as temporal variations in sunlight, temperature and humidity. To adapt this changing environment, almost all organisms on Earth have evolved circadian timing system that generates and regulates circadian rhythms in physiology, cellular and biochemical processes as well as in behaviors, such as body temperature, cell metabolism, hormone release, and sleep-wake cycle in mammals (Paschos et al., 2010, Feng and Lazar, 2012, Thaiss et al., 2015). The term “circadian” is derived from the Latin word “*circa diem*” that means “about a day”. Preservation of circadian rhythms is essential for human health. Chronic disruption of circadian rhythms is linked to a variety of pathogenic conditions including metabolic syndromes, inflammatory and cardiovascular diseases and cancers (Table 1) (Gery and Koeffler, 2010; Maury et al., 2010; Portaluppi et al., 2012; Mattis and Sehgal, 2016; Germain and Kupfer, 2008).

The circadian clock system consists of three main components (Figure 1): 1) the external inputs such as light, oxygen level and temperature that provide time cues (so-called time givers or zeitgebers); 2) the central clock (pacemaker) that senses the input

signals; and 3) the output pathways (or effector pathways) through which the central clock generates and maintains biological rhythms (Gaspar et al., 2019; Takahashi, 2017). In mammals, the central clock is located in the suprachiasmatic nucleus (SCN) of the hypothalamus and is also called master clock. Molecular clocks presented in other tissues/organs are called peripheral or slave clocks (Yoo *et al.,* 2004). The central clock synchronizes peripheral clocks via neural and hormonal pathways although feedback from the periphery to SCN is also possible (Mrosovsky, 1996). It is noteworthy that circadian oscillations can be self-sustained (independent of SCN) in peripheral tissues (Yoo *et al.,* 2004).

All molecular clocks consist of over 15 circadian genes that form multiple transcriptional-translational feedback loops (TTFLs) (Figure 1) (Feng *et al.,* 2012; Takahashi, 2017). In the main TTFL, Bmal1 and Clock form a heterodimer that activates transcription of target genes including *Pers* (periods) and *Crys* (crytochromes) via E-box *cis*-element (Bass and Takahashi, 2010; Takahashi, 2017). As the protein levels increase, Pers and Crys inhibit the activity of Bmal1/Clock to lower the expression of themselves and others, thereby generating a circadian oscillation in gene expression (Bass and Takahashi, 2010; Takahashi, 2017). A new transcriptional-translational cycle can be initiated when Pers and Crys are reduced to a low level due to protein degradation via phosphorylation and ubiquitination (Bass and Takahashi, 2010; Curtis *et al.,* 2014).

The second TTLF is composed of three transcriptional activators (Rorα, β, γ) and two repressors (Rev-erbα/β) (Figure 1) (Liu *et al.,* 2008). Rors and Rev-erbs compete

for binding to the same *cis*-element [named RORE (Ror response element) or RevRE (Rev-erb response element)] (Preitner *et al.,* 2002). Rors induce while Rev-erbs inhibit the transcription of target genes including *Bmal1* (Preitner *et al.,* 2002). The third TTLF is driven by Dbp and E4bp4. Dbp and E4bp4 compete for binding to the same DNA motif (called "D-box"), playing an antagonistic role in regulating expression of target genes including *Per2* (Dbp activates whereas E4bp4 represses gene transcription) (Mitsui *et al.,* 2001).

Regulation of drug-metabolizing enzymes by circadian clock genes

In general, circadian gene expression is generated by a transcriptional mechanism in which core clock genes act on three *cis*-elements (E-box, D-box and RORE or RevRE) in target gene promoter (Figure 2) (Takahashi, 2017; Zhao et al., 2019). These *cis*-elements generate a difference in the phase (peak timing) of circadian gene expression (Minam et al., 2013). Peak timing of D-box-driven and RORE-driven expression are respectively ~5 h and ~13 h delayed as compared with E-box-driven expression (Minam et al., 2013). Of circadian clock proteins, Bmal1 and Clock act on E-box, Dbp and E4bp4 act on D-box, whereas Rors and Rev-erbs act on RORE. There is accumulating evidence that these clock proteins (alone or in combination) generate and regulate diurnal rhythms of drug-metabolizing enzymes (Figure 2).

Bmal1 and Clock

Bmal1 and Clock, the positive elements of the main TTFL in circadian clock, are indispensable to generate circadian gene expression (Takahashi, 2017). Bmal1 and

Clock have been implicated in regulation of drug metabolism, contributing to time-varying drug exposure and toxicity. Bmal1 and Clock activate transcription of *Cyp2a4/5* via direct binding to E-box *cis*-elements in promoters (Zhao et al., 2019). Accordingly, knockout of Clock or Bmal1 down-regulates Cyp2a4/5 expression in mice (Zhao et al., 2019; Hatanaka et al., 2010). Clock ablation sensitizes mice to the toxicity of coumarin, a drug detoxified by Cyp2a4/5 (Zhao et al., 2019). *Fmo5* is a circadian gene that is under the control of Bmal1. Bmal1 regulation of Fmo5 is attained through direct binding to an E-box and transcriptional activation (Chen et al., 2019a). Bmal1 or Clock ablation leads to downregulation of Fmo5 expression and loss of diurnal rhythm in mouse liver (Chen et al., 2019a). *Ugt1a1* (containing a functional E-box in its promoter) is a direct target of Bmal1 (Wang et al., 2019). Bmal1 knockout decreases mRNA and protein expression of Ugt1a1 and blunts their circadian rhythms in mouse liver (Wang et al., 2019). This is accompanied by a loss of circadian time-dependency in bilirubin clearance and a higher sensitivity of mice to chemical-induced hyperbilirubinemia (Wang et al., 2019).

In addition to a direct transcriptional mechanism, Bmal1/Clock may regulate expression of drug-metabolizing enzymes through an indirect mechanism. Bmal1 regulates diurnal expression of Cyp3a11 through Dbp and Hnf4α, two direct targets of Bmal1 and activators of Cyp3a11 (Lin et al., 2019a). Bmal1 deficiency decreases Cyp3a11 expression, and abrogates the daily rhythm of Cyp3a11 expression in mouse liver and small intestine (Lin et al., 2019a, b). Also, Bmal1 ablation sensitizes mice to toxicities of Cyp3a11 substrate drugs (such as aconitine, hypaconitine and triptolide),

and blunts the rhythmicity in toxicity due to elevated drug exposure (Lin et al., 2019a, b). Consistently, deletion of Clock or Npas2 (performing similar functions as Clock does in some tissues) in mice reduces Cyp3a11 expression and aggravates the toxicities induced by triptolide and brucine (Zhou et al., 2019a). Clock represses Cyp2b10 transcription through Rev-erbα/β, two target genes of Clock and repressors of Cyp2b10. Clock ablation up-regulates Cyp2b10-mediated metabolism of cyclophosphamide (CPA) (a metabolic pathway generating the toxic metabolite 4-hydroxy-CPA), leading to exacerbated CPA toxicity and loss of chronotoxicity (Zhao et al., 2019). However, the chronotoxicity may be not solely attributed to circadian metabolism and pharmacokinetics because it is also correlated with diurnal sensitivity of target B cells regulated by Bmal1/Clock (Gorbacheva et al., 2005).

Bmal1 is also involved in the regulation of drug transporters and chronotoxicity. The cardiac glycoside oleandrin displays dosing time-dependent toxicity [ZT2 > ZT10 (ZT, zeitgeber time in a 12 h light and 12 h dark cycle; ZT0 represents lights on and ZT12 represents lights off)] in mice that is positively associated with the level of drug exposure (ZT2 > ZT10) (Zhou et al., 2019b). Intestinal ablation of Bmal1 increases the sensitivity of mice to oleandrin-induced toxicity and abolishes the toxicity rhythmicity (Zhou et al., 2019b). This is because oleandrin is a good substrate transported by P-gp, whose expression and rhythmicity are under the control of Bmal1 (Zhou et al., 2019b). In addition, diurnal expression of intestinal P-gp is a contributor to circadian responses of animals to irinotecan (Filipski et al., 2014). Mechanistically, Bmal1 regulates diurnal P-gp expression through activating Hlf (a positive regulator of P-gp)

and suppressing E4bp4 (a negative regulator of P-gp) (Zhou et al., 2019b).

Bcrp (Abcg2) is rhythmically expressed in mouse liver, kidney and intestine (Zhang et al., 2009; Hamdan et al., 2012). As a result, the pharmacokinetic behavior of oral sulfasalazine (a Bcrp substrate) is significantly influenced by dosing time (drug exposure: ZT2 > ZT14) (Hamdan et al., 2012). Clock deficiency decreases Bcrp expression and abolishes its rhythm in mouse small intestine (Hamdan et al., 2012). Mechanistic studies reveal that Clock regulates Bcrp through circadian clock-activating transcription factor-4 that periodically binds to Bcrp promoter and activates gene transcription (Hamdan et al., 2012).

Mrp2 expression varies greatly with the times of the day in both mouse liver and intestine, accounting for diurnal elimination and toxicity of Mrp2 substrates such as bilirubin, phenolsulfonphthalein, methotrexate and irinotecan (Wang et al., 2019; Oh et al., 2017; Yu et al., 2019; Okyar et al., 2011), Mrp2 mRNA and protein increase in the dark phase and decrease in the light phase in both mouse liver and intestine. Accordingly, hepatobiliary excretion of phenolsulfonphthalein is greater in mice when administered during the dark phase (higher Mrp2 expression) than during the light phase (lower Mrp2 expression) (Oh et al., 2017). Bmal1 has been reported to regulate diurnal expression of Mrp2 in mouse liver and intestine. Loss of Bmal1 decreases Mrp2 expression and blunts the rhythmicity, leading to increased sensitivity of mice to toxicity induced by bilirubin and methotrexate (Wang et al., 2019, Yu et al., 2019). Bmal1 activates Mrp2 transcription via up-regulating Dbp (an Mrp2 activator) expression and down-regulating E4bp4 (an Mrp2 repressor) expression through Rev-erbα (an E4bp4

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repressor) (Figure 3) (Yu et al., 2019).

Dbp and E4bp4

Dbp and E4bp4 are two transcriptional factors that compete for binding to the same DNA sequence (called D-box) in target gene promoter (Mitsui et al., 2001). Dbp activates whereas E4bp4 inhibits gene transcription, thereby playing an antagonistic role in regulating gene expression (Mitsui et al., 2001). Reported common target genes of Dbp and E4bp4 are involved in circadian regulation and xenobiotic disposition (Table 2). Dbp and E4bp4 have been identified as important circadian regulators of drug-eliminating genes and chronotoxicity. Dbp and other two PAR bZIP proteins (Tef and Hlf) may regulate a number of drug-metabolizing enzymes including Cyp2a, Cyp2c and Ces3 (Gachon et al., 2006). In particular, the PAR bZIP proteins indirectly regulate diurnal expression of Cyp2b10 through Car (constitutive androstane receptor). Dbp/Tef/Hlf triple knockout mice show an increased susceptibility to toxicity induced by mitoxantrone and CPA, two Cyp2b10 substrates (Gachon et al., 2006). Dbp binds to the promoters of *Cyp2a4* and *Cyp2a5* and regulates their circadian expression in the mouse liver (Lavery et al., 1999). Consistently, E4bp4 represses *Cyp2a5* transcription by binding to a D-box located at -924/-904 bp, and Shp promotes Cyp2a5 expression via suppressing E4bp4 activity (Zhang et al., 2018). Moreover, E4bp4 positively regulate Ces2 expression by inhibiting the activity of Rev-erbα, a transcriptional repressor of Ces2 (Zhao et al., 2018). Loss of E4bp4 decreases Ces2 expression and activity in mouse liver, resulting in reduced clearance and improved bioavailability of

CPT-11 (a Ces2 substrate) (Zhao et al., 2018).

DBP and E4BP4 regulate diurnal expression of human CYP3A4. CYP3A4 mRNA, protein and enzymatic activity show temporal rhythmicities in serum-shocked HepG2 cells (Takiguchi et al., 2007). DBP binds to a D-box element (located at -34/-24 bp) in *CYP3A4* promoter and activates its transcription, while E4bp4 antagonizes such effect (Takiguchi et al., 2007). Overexpression of DBP increases CYP3A4 mRNA expression, while overexpression of each of other circadian clock genes (i.e., *PER2*, *CRY1* and *REV-ERBα*) has no effects (Takiguchi et al., 2007). In addition, Dbp and E4bp4 regulate diurnal Cyp3a11 (the orthologue of human CYP3A4) expression in mouse liver. Dbp binds to a D-box at -45/-36 bp in *Cyp3a11* promoter and activates its transcription, whereas E4bp4 binds to a D-box at -1539/-1529 bp and represses gene transcription (Lin et al., 2019a; Tong et al., 2019). E4bp4 ablation reduces the systemic exposure of midazolam (a specific Cyp3a11 substrate) in mice through promoting its metabolism by Cyp3a11 (Tong et al., 2019).

Dbp and E4bp4 are also involved in the regulation of diurnal Fmo5 expression and circadian pharmacokinetic of Fmo5 substrates. Fmo5 mRNA, protein and activity display robust rhythmicity in mouse liver, accounting for dosing time-dependent pharmacokinetic profiles of pentoxifylline (an Fmo5 substrate) (Chen et al., 2019a). Deletion of E4bp4 increases hepatic Fmo5 expression and blunts its rhythms in mice (Chen et al., 2019a). In fact, Fmo5 promoter contains two D-boxes (located at -1718 bp and -796 bp). E4bp4 acts on both D-boxes, whereas Dbp acts only on the latter D-box (-796 bp) (Chen et al., 2019a).

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P-gp expression displays a robust fluctuation in multiple tissues, including the liver, intestine and kidney (Ando et al., 2005). E4bp4 and PAR bZIP factors (i.e., Dbp, Tef and Hlf) participate in circadian regulation of P-gp expression. E4bp4 represses, whereas Hlf activates *mdr1a* transcription via competitive binding to a D-box element (please note that mouse P-gp is encoded by *mdr1a*, *mdr1b* and *mdr2* genes) (Zhou et al., 2019b). *Mdr2* promoter also contains a functional D-box through which PAR bZIP factors activate and E4bp4 inhibits gene transcription (Kotaka et al., 2008). Diurnal expression of intestinal P-gp has been shown to be a critical factor influencing daily exposure and toxicity of P-gp substrates such as oleandrin and digoxin (Zhou et al., 2019b; Ando et al., 2005). Quinidine (a P-gp substrate) exposure in brain tissue varies according to the time of administration (Kervezee et al., 2014). This time difference is lost upon P-gp inhibition (Kervezee et al., 2014). In addition, Dbp and E4bp4 paly a mediating role in Bmal1 regulation of Mrp2 rhythm (Figure 3). Dbp and E4bp4 are the target genes of Bmal1 and regulators of Mrp2 (Yu et al., 2019). They bind to a same D-box (-100/-89 bp) element in *Mrp2* promoter in a time-dependent manner. The former activates while the latter represses *Mrp2* transcription (Yu et al., 2019).

Rev-erbs and Rors

Rev-erbs (Rev-erbα, β) and Rors (Rorα, β, γ) are transcriptional factors that compete for binding to a specific DNA sequence [named RevRE or RORE, generally composed of a NR half site (AGGTCA) and a preceding 5-bp A/T-rich sequence], thereby regulating gene transcription and expression (Harding and Lazar, 1993; Harding and

Lazar, 1995). Although binding to the same sequence, Rev-erbs and Rors generate opposite effects. The former inhibits while the latter activates target gene transcription. Transcriptional repressor activities of Rev-erbs are associated with enhanced recruitment of Ncor1 (nuclear receptor corepressors 1) and Hdac3 (histone deacetylase 3) complex to target gene promoter (Zamir et al, 1996; Yin et al., 2005). It is noted that Rev-erbs and Rors are expressed in a tissue-dependent manner (Yang et al., 2006). The ratios between Rev-erbs and Rors are a key determinant to circadian gene expression, providing a mechanism to fine-tune the circadian network and metabolism (Yang et al., 2006).

Rev-erbs and Rors have been identified as regulators of drug-metabolizing genes, impacting circadian metabolism and chronopharmacokinetics. The mRNA expression levels of six Ugt2b genes (i.e., Ugt2b1, Ugt2b5, Ugt2b35, Ugt2b36, Ugt2b37, and Ugt2b38) show circadian fluctuations in mouse liver (Zhang et al., 2019a). Likewise, total Ugt2b protein and activity toward morphine exhibit a circadian rhythm in the liver (Zhang et al., 2019a). Loss of Rev-erbα increases hepatic Ugt2b expression and blunts its rhythm in mice (Zhang et al., 2019a). Mechanistically, Rev-erbα trans-represses Ugt2b genes via direct binding to a RevRE element, generating a diurnal rhythmicity in Ugt2b expression (Zhang et al., 2019a). Interestingly, Shp blocks the suppressive effects of Rev-erbα on Ugt2b and modulates morphine metabolism and morphine withdrawal syndrome (Chen et al., 2019b). In addition, Rev-erbα contributes to diurnal expression of Cyp2b10, Cyp4a10 and Cyp4a11 through transcriptional actions on RevRE elements (Zhang et al., 2018). Shp prevents the recruitment of co-repressors

Ncor1/Hdac3 to Rev-erbα, leading to de-repression of these Cyp genes (Zhang et al., 2018). Rev-erbα is also a transcriptional repressor of Ces2. Overexpression of Rev-erbα represses Ces2 expression, whereas knockdown of Rev-erbα increases Ces2 expression (Zhao et al., 2018). By acting on the target gene *E4bp4*, Rev-erbα participates in circadian regulation of the metabolic enzymes such as Fmo5 and Cyp7a1 (Duez et al., 2008; Chen et al., 2019a).

RORα and RORγ regulate expression of human CYP2C8 as knockdown of RORα or RORγ decreases the mRNA level of CYP2C8 in HepG2 cells (Chen et al., 2009). A RORE element located at -2045 bp is identified in *CYP2C8* promoter to be essential for ROR-mediated transactivation (Chen et al., 2009). Also, RORα and RORγ regulate SULT2A1 expression through direct binding to a RORE element in the proximal gene promoter (Ou et al., 2013). Supporting this, SULT2A1 expression is positively correlated with RORα/γ expression in primary human hepatocytes and in human livers (Ou et al., 2013). Additionally, overexpression of Rorα stimulates Cyp3a11 expression, although the underlying mechanism remains unknown (Wada et al., 2008).

Circadian regulation of drug-metabolizing enzymes by nuclear receptors (NRs)

Nuclear receptors (NRs) are a class of transcription factors and most of them are ligand-responsive (can be activated by a variety of endogenous and exogenous chemicals) (Belandia and Parker, 2003; Mangelsdorf et al., 1995). In general, a NR protein possesses four modular domains (Figure 4A): a highly variable N-terminal region which may harbor an activation function (AF-1), a DNA binding domain

containing two zinc-finger motifs, a flexible hinge domain, and ligand binding domain that harbors an activation function (AF-2). Some NRs such as CAR and PXR (pregnane X receptor) work by forming a heterodimer with RXR (retinoid-X receptor) (Evans and Mangelsdorf, 2014). The heterodimers bind to specific DNA motifs (repeats of nucleotide hexamer AGG/TTCA with a variable spacing) and regulate gene transcription (Figure 4B).

Expression levels of drug-metabolizing enzymes and transporters are under the control of many NRs (Tolson and Wang, 2010; Chen et al., 2012; Li et al., 2019), including CAR, PXR, RXR, PPARs (peroxisome proliferator activated receptors), farnesoid X receptor, LXRs (liver X receptors), VDR (vitamin D receptor), HNF4α (hepatocyte nuclear factor 4 alpha), LRH-1 (liver receptor homolog 1) and SHP (small heterodimer partner) (Figure 4C). At the same time, these NRs may be circadian clock-controlled proteins (called “cycling NRs”) whose expression levels oscillate with the times of the day (Yang et al., 2006). The rhythms of cycling NRs can be propagated to the downstream target genes. The rhythmicity generated via clock output genes is essentially an indirect mechanism as compare to direct regulation by circadian clock genes.

Cycling NRs

Of drug metabolism-related NRs, Pxr, Hnf4α, Shp, Ppar-α, and Ppar-γ mRNAs display strong diurnal oscillations (the peak to valley ratio >2) in mouse liver (Zhang et al., 2009; Oiwa et al., 2007; Yang et al., 2006). These mRNAs generally peak in the late

light phase. Hnf4α and Ppar-γ proteins oscillate with times of the day (Lin et al., 2019a; Deng et al., 2018). The phase of diurnal Ppar-γ is shifted about 4 h due to a potential delay in the translation of mRNA to protein product (Figure 5) (Deng et al., 2018). Large phase shifts (about 8-12 h) between protein and mRNA are also observed for Cyp2e1 and Cyp3a11 as well as Bmal1 and Clock (Figure 5) (Zhang et al., 2018; Lin et al., 2019a). By contrast, there is no mRNA-protein phase shift for clock genes such as Rev-erbα, Dbp, E4bp4 and Per2 (Figure 5) (Narumi et al., 2016). Fxr, Vdr, Lxr, Lrh-1 and AhR show mild or weak fluctuations in mRNA expression (Zhang et al., 2009; Tanimura et al., 2011; Lin et al., 2019a). There are conflicting data regarding diurnal expression of Car in the liver. Wu and coworkers reports no circadian time-dependent variations in *Car* mRNA consistent with a prior study although intestinal Car may be diurnally rhythmic (Lin et al., 2019a; Kawase et al., 2013). However, Gachon et al (2006) show that rhythmic Car in the liver mediates regulation of Cyp2b10 by three PAR bZIP factors.

Unfortunately, the mechanisms for circadian expression of most cycling NRs are poorly understood. However, studies have been performed to explore how Hnf4α and Shp rhythms are generated. Bmal1 is a source of Hnf4α rhythm as loss of Bmal1 reduces Hnf4α expression and abrogates its rhythm in mouse liver (Lin et al., 2019a). Bmal1 regulation of Hnf4α is attained through two E-boxes in the distal region (from −6.1 to −6.0 kb) of P1 promoter (Lin et al., 2019a). Bmal1, Clock, Naps2 and Rev-erbα are potential contributors to circadian expression of Shp. They regulate *Shp* transcription via binding to the E-box or RevRE element (Oiwa et al., 2007; Duez et al.,

2008; Pan et al., 2010).

Cycling NR-regulated enzymes

Car perhaps is the first reported cycling NR that regulates circadian expression of a metabolic enzyme (Cyp2b10). Rhythmic Car transcriptionally drives transcription of Cyp2b10 via the phenobarbital-response element, thereby generating a diurnal rhythmicity in Cyp2b10 expression (Ripperger et al., 2006; Gachon et al., 2006). Hnf4α is another cycling NR that contributes to enzyme rhythmicity. Cyp3a11 rhythm has been shown to be partly associated with direct regulation of Hnf4α via a DR1 element (Lin et al., 2019a). Diurnal Ppar-γ protein level is significantly correlated with circadian *Cyp2a5* mRNA level (Deng et al., 2018). The latter presents a PPRE element in gene promoter through which the former activates gene transcription (Deng et al., 2018). These data support a contribution of Ppar-γ to generation of Cyp2a5 rhythm.

Shp has been implicated in circadian regulation of Cyp enzymes (including Cyp1a2, Cyp2a5, Cyp2b10, Cyp2c38, Cyp2c39, Cyp2e1, Cyp3a11, Cyp4a10 and Cyp4a14) via crosstalk with multiple circadian proteins (Dec2, E4bp4, Rev-erbα, and Lrh1/Hnf4α) (Zhang et al., 2018). Of note, Shp ablation blunts the circadian rhythmicity in acetaminophen-induced hepatotoxicity in mice and alleviates the toxicity by down-regulating Cyp2e1-mediated metabolism and reducing formation of the toxic metabolite (Zhang et al., 2018). Rhythmic AhR partially accounts for diurnal expression of Cyp1a1 and Cyp1b1 (Huang et al., 2002). AhR-mediated induction of Cyp1a1 depends on the time of dioxin (an AhR agonist) administration with the highest extent

of induction occurring in the nighttime (Huang et al., 2002). Additionally, we recently found that the NR corepressor RIP140 is rhythmically expressed in the liver, and loss of RIP140 dampens the rhythm of Cyp2b10 (unpublished data). This may highlight a complexity in the mechanisms for generation of circadian gene expression.

Rhythmic patterns for drug-metabolizing enzymes

Current literature reveals two modes (i.e., a general mode and an alternative mode) for generation of diurnal rhythmicity in drug-metabolizing enzymes. In the general mode, circadian clock genes generate and maintain diurnal gene expression via transcriptional actions on one or two of three cis-elements (i.e., E-box, D-box, and RevRE or RORE) (Figure 2). The alternative mode involves cycling NRs such as Hnf4α and Ppar-γ. The rhythms of cycling NRs are propagated to the downstream target genes many of which are drug-processing genes. The general mode tends to produce two types of diurnal patterns for mRNA expression, namely, a convex pattern (Figure 6A) and a concave pattern (Figure 6B). The convex pattern (e.g., *Cyp2e1* and *Cyp3a11* mRNAs) is characterized by higher expression in the daytime and lower expression in the nighttime with a peak value in the late light phase (Figure 6A). The concave pattern (e.g., *Cyp2b10* mRNA) is characterized by higher expression in the nighttime and lower expression in the daytime with a trough value in the late light phase (Figure 6B). The mRNA patterns (e.g., *Cyp2a5* mRNA) deviated from the above two typical curves may result from rhythmic modifications from cycling NRs for which translation from mRNA to protein is significantly delayed (e.g., Ppar-γ).

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Metabolism-based chronotoxicity

Cyp3a11-mediated chronotoxicity

Mouse Cyp3a11 (CYP3A4 in humans) is one of the most important enzymes responsible for drug metabolism and detoxification. The role of Cyp3a11 in determining drug chronotoxicity has been well established. Cyp3a11 protein varies according to the times of the day with higher expression during the nighttime and lower expression during the daytime (Lin et al., 2019a). As a result, drugs (e.g., aconitine, triptolide, and brucine) detoxified by Cyp3a11 are more toxic to mice in the daytime than in the nighttime (Lin et al., 2019a, b; Zhou et al., 2019a). In addition, diurnal expression of Cyp3a11 accounts for chronotoxicity of herbal medicines such as *Fuzi* (lateral root of *Aconitum carmichaeli*) and *Tripterygium wilfordii* (Figure 7A) (Yang et al., 2020). Mice are more sensitive to *Fuzi* or *Tripterygium wilfordii* (oral gavage) in the light phase than in the dark phase as the toxic ingredients are detoxified by Cyp3a11 (Figure 7A) (Yang et al., 2020).

Cyp2e1-mediated chronotoxicity

Mouse Cyp2e1 protein shows a diurnal pattern in the liver similar to that of Cyp3a11 (higher levels in the nighttime and lower levels in the daytime) (Zhang et al., 2018). Acetaminophen (APAP) toxicity exhibits circadian rhythmicity in wild-type mice. APAP injected at ZT14 (dark phase) induces a higher level of toxicity compared with ZT2 (light phase) (Zhang et al., 2018). The chronotoxicity of APAP is attributed to circadian Cyp2e1 that generates the toxic metabolite N-acetyl-p-benzoquinone imine from APAP

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(Zhang et al., 2018). More severe toxicity is thus associated with a higher expression of Cyp2e1.

Chronotoxicity mediated by other Cyp enzymes

Coumarin hepatotoxicity displays a diurnal rhythmicity in mice (the toxicity is more severe at ZT2/22 than that at ZT14) (Zhao et al., 2019). The diurnal pattern of toxicity is anti-phase to that of Cyp2a4/5, two enzymes primarily responsible for detoxification of coumarin (Zhao et al., 2019). CPA is a prodrug and bioactivated by Cyp2b10 to 4-OH-CPA (the active and toxic form) in mice. The severity of CPA toxicity in mice is dosing time-dependent with higher levels at ZT2/22 and lower levels at ZT10/14 (Zhao et al., 2019). This results from a diurnal rhythmicity in hepatic Cyp2b10 protein (higher levels at ZT2/22 and lower levels at ZT10/14).

Transporter-based chronotoxicity

Zhou et al (2019b) have reported circadian time-dependent responses of mice to the cardiac glycoside oleandrin, a P-gp substrate. Mice treated during the times of dark-to-light transition (ZT22 to ZT2) are more sensitive to the drug than the mice treated in the late light phase (ZT10) (Zhou et al., 2019b). This time-dependent sensitivity is correlated with the daily variations in drug exposure caused by diurnal expression of intestinal P-gp. Methotrexate is an inhibitor of dihydrofolate reductase and used to treat neophastic cancers and autoimmune diseases. Oral methotrexate is more toxic in the early dark period (ZT14) than in the early morning period (ZT2) in mice (Yu et al., 2019). This chronotoxicity is mainly dependent on the circadian rhythm of Mrp2 expression. A

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lower level of toxicity at ZT2 is associated with a higher Mrp2 expression (and a lower drug absorption) and a higher level of toxicity at ZT14 with a lower Mrp2 expression (Yu et al., 2019). We also observe a diurnal rhythmicity in the toxicity of *Semen Strychni* that is mainly accounted for by circadian intestinal efflux transport although circadian hepatic metabolism may also play a role (Figure 7B).

Chronoefficacy

Theoretically, circadian metabolism would result in time-varying drug efficacy (chronoefficacy) in addition to chronotoxicity due to diurnal variations in drug exposure. However, the experimental evidence is still lacking in metabolism-based chronoefficacy. Contrasting with this, there is accumulating evidence that diurnal rhythms of disease severity and drug target can be linked to chronoefficacy (Ruben et al., 2019; Bass and Lazar, 2016). Studies with animals have revealed that drug efficacy could be improved by altering the dosing time according to the expression of clock-controlled drug targets or transporters (Table 3). Rhythmicity in disease severity may involve a circadian disease regulator (e.g., clock genes). The clock gene *Rev-erbα* has been implicated in regulation of colitis via NF-κB/Nlrp3 axis, generating a diurnal rhythmicity in the severity of inflammations (Figure 8A) (Wang et al., 2018). Zhou et al (2020) uncover a time-varying berberine (a Rev-erbα agonist) effect on chronic colitis in mice (Figure 9A). ZT10 dosing generates higher therapeutic efficacy (reflected by lower levels of inflammatory markers) compared to ZT2 dosing (Figure 9A, Zhou et al., 2020). The time-varying berberine effects are accounted for by diurnal rhythmicities in both colitis severity and drug target (Rev-erbα) (Zhou et al., 2020). A superior efficacy at ZT10 is

associated with less severe colitis and a higher Rev-erbα expression (Zhou et al., 2020). The authors propose a dual role for Rev-erbα in regulation of time-varying berberine effect, namely, generating diurnal rhythmicity in colitis and acting as rhythmic drug target (Zhou et al., 2020).

Rev-erbα has been also implicated in regulation of homocysteine homeostasis via three catabolic enzymes (Bhmt, Cbs and Cth), generating a diurnal rhythmicity in body homocysteine (Figure 8B) (Zhang et al., 2019b). Most recently, Chen et al (2020) reveal the time-varying effects of the Rev-erbα antagonist puerarin on hyperhomocysteinemia in mice (i.e., puerarin treated at ZT10 shows a stronger effect than puerarin treated at ZT22) (Figure 9B). The circadian effects of puerarin on hyperhomocysteinemia are accounted for by rhythmic Rev-erbα that is identified as the drug target of puerarin (Chen et al., 2020). The cholesterol-lowing effects of short-acting statins (e.g., fluvastatin, simvastatin, lovastatin and pravastatin) in humans depend on the time of administration (evening > morning) (Awad and Banach, 2018). This is probably because the drug target HMG-CoA (3-hydroxy-3-methyl glutaryl coenzyme A) reductase is expressed at higher levels in the nighttime (Jones and Schoeller, 1990).

Concluding remarks

Many drug-metabolizing enzymes in mice have been identified to be rhythmically expressed in the liver and intestine. By contrast, a very limited number of human CYP genes (i.e., CYP2D6 and CYP3A4) are characterized as circadian genes *in vitro*. Extensive studies with cells and mice in recent years have revealed two modes (i.e., a

general mode and an alternative mode) for generation of diurnal rhythmicity in drug-metabolizing enzymes. In the general mode, circadian clock genes generate and regulate diurnal gene expression via transcriptional actions on one or two of three *cis*-elements (i.e., E-box, D-box, and RevRE or RORE). The alternative mode involves cycling NRs such as Hnf4α and Ppar-γ. The rhythms of cycling NRs can be propagated to the downstream target genes many of which are drug-processing genes.

The rest-activity cycle is inverted between humans (diurnal creatures) and rodents (nocturnal species). This may raise serious concerns about whether the circadian mechanisms for drug metabolism could be translated from rodents to humans. However, the basic mechanisms for circadian clock and for circadian gene expression are thought to be well conserved in mammals. Although the diurnal patterns of mouse drug-processing genes cannot be directly mapped to those of human counterparts, the regulatory relationships of circadian oscillators with their targets should be preserved between humans and mice. Future studies are suggested to validate the discovered circadian mechanisms in mice for drug-processing genes using human-derived cells and primates. These studies are useful in attempt to predicting circadian patterns of drug-processing genes in humans.

Theoretically, circadian metabolism would result in chronoefficacy in addition to chronotoxicity due to diurnal variations in drug exposure caused by circadian metabolism. Contrasting with well-established relationships of circadian metabolism and pharmacokinetics with chronotoxicity, the experimental evidence is still lacking in metabolism-based chronoefficacy. This is probably because very few or no studies

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were ever performed to examine both circadian metabolism and chronoefficacy. Such studies appear to be essential in order to advance drug chronotherapeutics because best timing for drug administration should be derived by taking both drug toxicity and efficacy into consideration.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Lu, Zhao, Chen and Wu.

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Footnotes

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Figure legends

Figure 1 Circadian clock system in mammals. Mammalian circadian clock system consists of three main components: 1) the external inputs such as light, oxygen level and temperature that provide time cues (so-called time givers or zeitgebers); 2) the central clock (pacemaker) that senses the input signals; and 3) the output pathways (or effector pathways) through which the central clock generates and maintains biological rhythms. At the molecular level, clocks consist of over 15 circadian genes that form multiple transcriptional-translational feedback loops (TTFLs). In the main TTFL, Bmal1 and Clock form a heterodimer that activates transcription of target genes including Pers and Crys via E-box cis-element. As the protein levels increase, Pers and Crys inhibit the activity of Bmal1/Clock to lower the expression of themselves and others, thereby generating a circadian oscillation in gene expression. A new transcriptional-translational cycle can be initiated when Pers and Crys are reduced to low levels due to protein degradation via phosphorylation and ubiquitination

Figure 2 General modes for generation of diurnal rhythmicity in drug-metabolizing enzymes and transporters through transcriptional actions on E-box, D-box and/or RevRE cis-elements.

Figure 3 Bmal1 regulates diurnal expression of Mrp2 through Dbp and Rev-erbα/E4bp4 pathways. To be specific, Bmal1 activates Mrp2 transcription via up-regulating Dbp (an Mrp2 activator) expression and down-regulating E4bp4 (an Mrp2 repressor) expression through Rev-erbα (an E4bp4 repressor).

Figure 4 Structure (A), DNA binding (B), and phylogeny (C) of nuclear receptors involved in regulation of drug-metabolizing enzymes and transporters.

Figure 5 Diurnal expression patterns of mRNA and protein for clock genes (Bmal1, Clock, Rev-erbα, Dbp, E4bp4 and Per2), nuclear receptor (Ppar-γ) and drug-metabolizing enzymes (Cyp2e1/3a11).

Figure 6 Representative diurnal convex pattern (A) and concave pattern (B) for rhythmic drug-metabolizing enzymes. The convex pattern (e.g., *Cyp2e1* and *Cyp3a11* mRNAs) is characterized by higher expression in the daytime and lower expression in the nighttime with a peak value in the late light phase. The concave pattern (e.g., *Cyp2b10* mRNA) is characterized by higher expression in the nighttime and lower expression in the daytime with a trough value in the late light phase.

Figure 7 Metabolism-based chronotoxicity of herbal medicines. (A) Diurnal expression of hepatic Cyp3a11 determines the chronotoxicity of *Fuzi* and

Tripterygium wilfordii in mice. Mice are more sensitive to *Fuzi* or *Tripterygium wilfordii* (oral gavage) in the light phase than in the dark phase as the toxic ingredients are detoxified by Cyp3a11. (B) Diurnal metabolism and efflux determines the chronotoxicity of *Semen Strychni*. Mice are more sensitive to *Semen Strychni* (oral gavage) in the dark phase than in the light phase as the toxic ingredients are detoxified by efflux transporter or drug-metabolizing enzymes.

Figure 8 Rev-erbα-based rhythmic diseases. (A) Rev-erbα regulates colitis via NF-κB/Nlrp3 axis. The clock gene *Rev-erbα* has been implicated in regulation of colitis via NF-κB/Nlrp3 axis, generating a diurnal rhythmicity in the severity of inflammations (Wang et al., 2018). (B) Rev-erbα regulates homocysteine homeostasis via three catabolic enzymes (Bhmt, Cbs and Cth). Rev-erbα directly binds to RevRE elements located in the promoters of Bhmt, Cbs and Cth and down-regulates their transcription, leading to elevated homocysteine level and decreased ammonia clearance (Zhang et al., 2019b).

Figure 9 Chronoefficacy of berberine (A) and puerarin (B) is associated with diurnal expression of Rev-erbα. Berberine (a Rev-erbα agonist) alleviates chronic colitis in mice in a dosing time-dependent manner (ZT10 > ZT2) consistent with diurnally rhythmic expression of intestinal Rev-erbα (a high expression at ZT10 and a low expression at ZT2). Puerarin (a Rev-erbα antagonist) alleviates hyperhomocysteinemia in mice in a dosing time-dependent manner (ZT10 > ZT22) consistent with diurnally rhythmic expression of hepatic Rev-erbα (a high expression at ZT10 and a low expression at ZT22).

Table 1

Pathologic conditions associated with chronic circadian disruption.

Pathogenic conditions	Consequences and potential mechanisms	References
Cancer	Circadian disruption promotes cancer progression through enhancing the stemness and tumor-initiating potential of tumor cells and creating an immunosuppressive shift in the tumor microenvironment	Hadadi et al., 2019
Diabetes	Circadian disruption accelerates type 2 diabetes mellitus through inducing pancreatic β-cell loss and dysfunction	Gale et al., 2011
Obesity	Circadian dysfunction increases the risk of obesity by disrupting leptin signaling in adipose tissue	Kettner et al., 2015
Nonalcoholic fatty liver disease	Circadian disruption increases the risk for nonalcoholic fatty liver disease that is associated with the perturbation in metabolism.	Shetty et al., 2018
Colitis	Circadian clock disruption exacerbates experimental colitis through regulation of Rev-erbα/NF-κB/Nlrp3 pathway	Wang et al., 2018
Inflammation	Chronic circadian disruption aggravates inflammatory responses due to increased release of proinflammatory cytokines in peritoneal macrophages	Castanon-Cervantes et al., 2010
Psychiatric disease	Disruption of circadian rhythms leads to learning, memory and cognitive defects through inducing neuron impairments	Karatsoreos, 2014

Table 2

Common Dbp/E4bp4 targets and their functions.

Targets	Functions	References
Per1	A circadian factor that forms a heterodimer with Crys to repress Clock/Bmal1 activity.	Mitsui et al., 2001
Arnt	A cofactor for AhR and HIF1 that regulate the expression of genes involved in xenobiotic metabolism.	Nakabayashi et al., 2013
Cyp2a5	A drug-metabolizing enzyme involved in the metabolism and detoxification of xenobiotics.	Lavery et al., 1999; Zhang et al., 2018
CYP3A4	A drug-metabolizing enzyme involved in the metabolism and detoxification of xenobiotics.	Takiguchi et al., 2007
Cyp3a11	A drug-metabolizing enzyme involved in the metabolism and detoxification of xenobiotics.	Lin et al., 2019a; Tong et al., 2019)
Cyp7a1	The rate-limiting enzyme that catalyzes the conversion of cholesterol to bile acids in the liver.	Noshiro et al., 2007
Fmo5	A NADPH-dependent flavoenzymes that catalyzes the oxidation of soft nucleophilic heteroatom centers in drugs, pesticides, and xenobiotics.	Chen et al., 2019a
Mrp2	An ABC transporter that mediates the efflux of endogenous/exogenous compounds.	Yu et al., 2019

Table 3

Examples of drugs with chronoefficacy and corresponding clock-controlled drug targets or transporters.

Drug name	Associated circadian protein	Models	Chronoefficacy	References
Sulfasalazine	Slc7a11	Mice with colon 26 xenograft	ZT10 > ZT22	Okazaki et al., 2017.
N,N-diethylaminobenzaldehyde	Aldh3a1	Mice with 4T1 xenograft	ZT14 > ZT2	Matsunaga et al., 2018
Erlotinib	EGFR	Mice with HCC827 xenograft	ZT8 > ZT20	Lin et al., 2015
Lapatinib	EGFR	Mice with N87 xenograft	ZT23 > ZT13	Lauriola et al., 2014
Imatinib	PDGFR	Mice with xenograft	ZT2 > ZT14	Nakagawa et al., 2006
Nutlin 3	p53	Tumor cells from UV.BAL-5.4G xenograft	ZT14 > ZT2	Horiguchi et al., 2013
Pregabalin	Octn1	Diabetic mice	ZT14 > ZT2	Akamine et al., 2015
Gabapentin	Calcium channel α2δ-1 subunit	Mice with partial sciatic nerve ligation	ZT22 > ZT10	Kusunose et al., 2010
Rivaroxaban	Factor X	Rats	ZT2 > ZT14	Fujiwara et al., 2017
RS102895	CCL2	Hypercholesterolemic mice	ZT17-ZT1 > ZT5-ZT13	Winter et al., 2018
Puerarin	Rev-erbα	Mice with hyperhomocysteinemia	ZT10 > ZT22	Chen et al., 2020
Berberine	Rev-erbα	Mice with chronic colitis	ZT10 > ZT2	Zhou et al., 2020

Figure 1

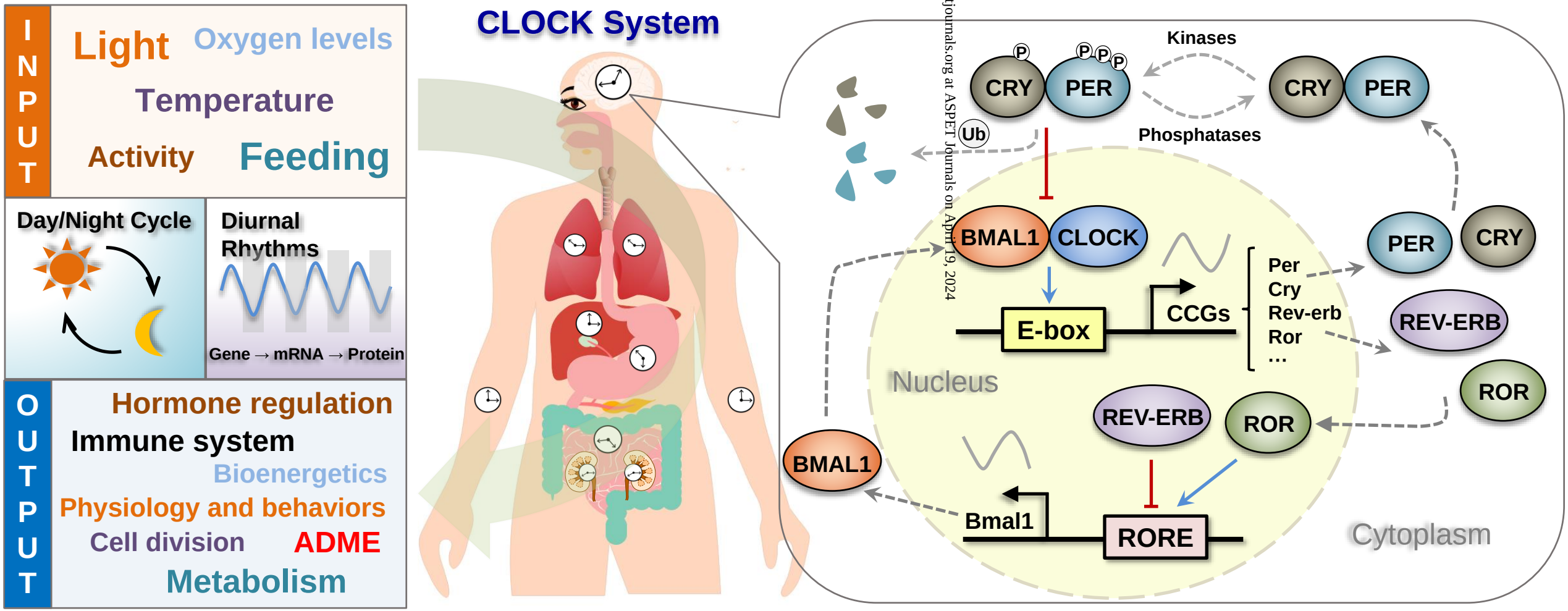


Figure 2

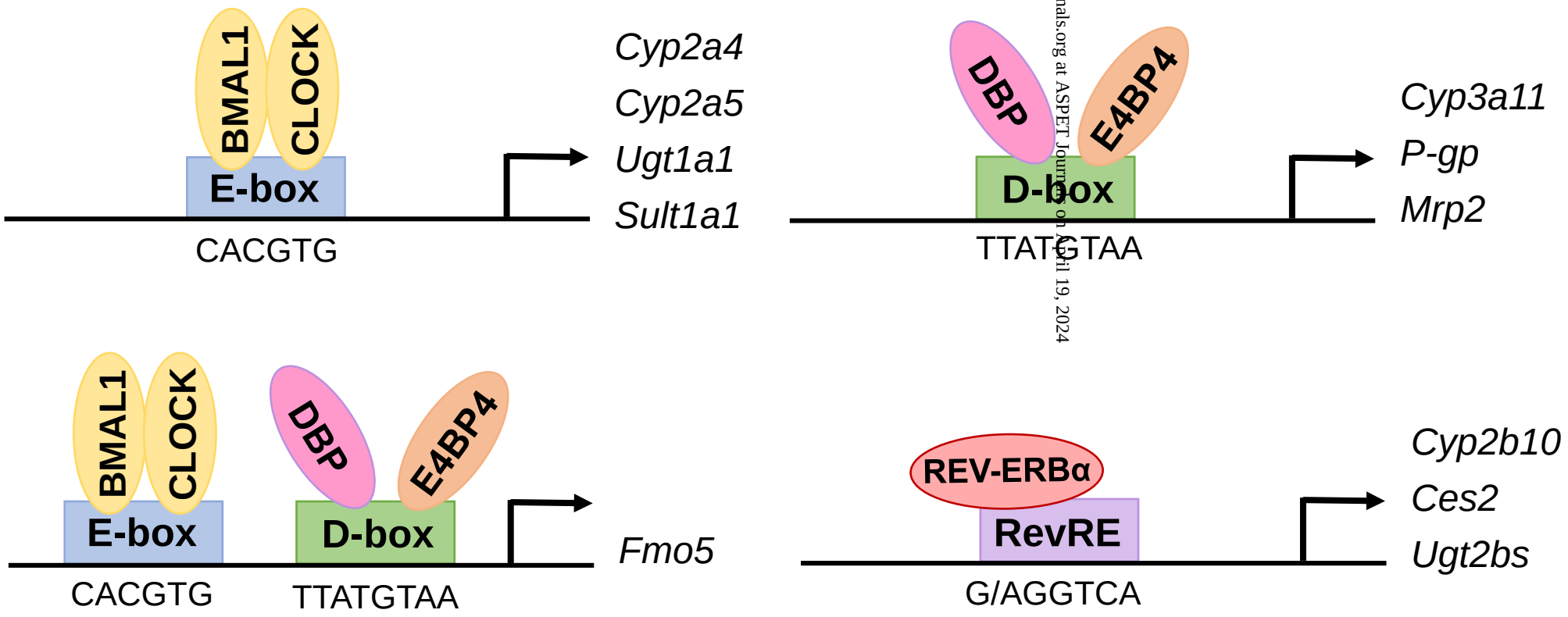


Figure 3

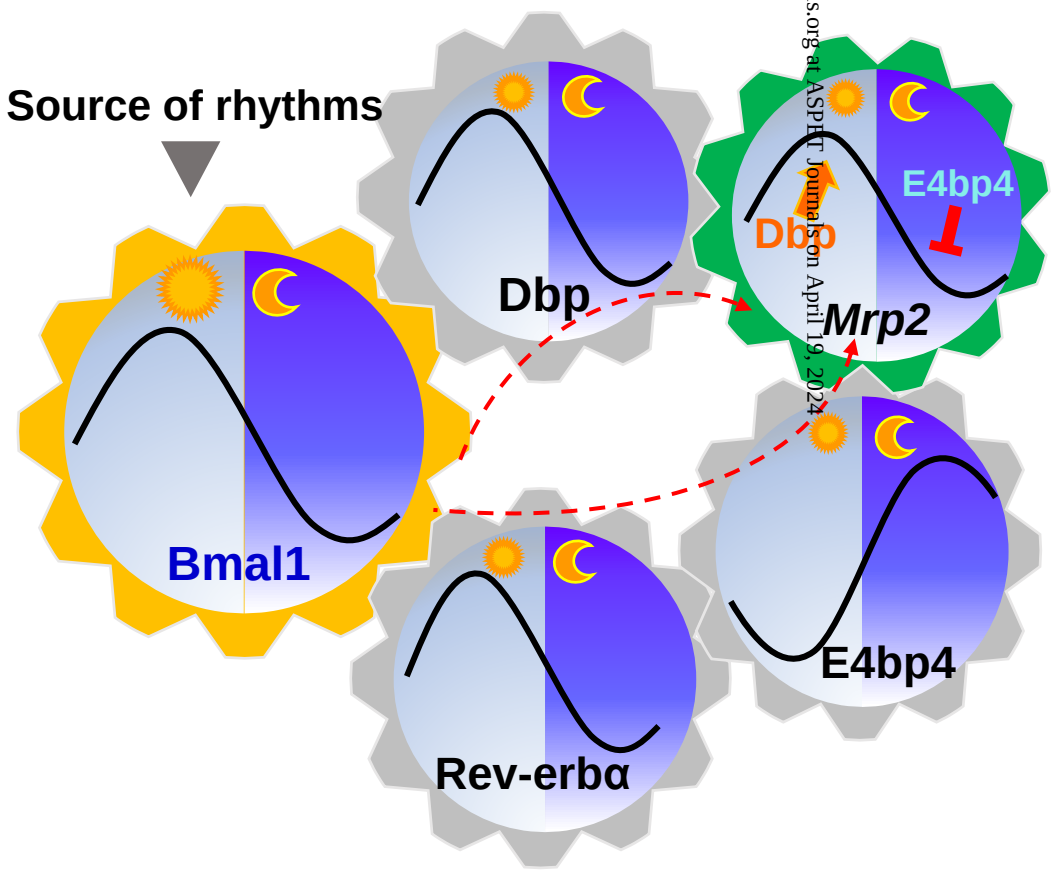
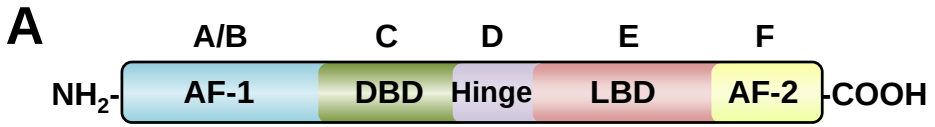
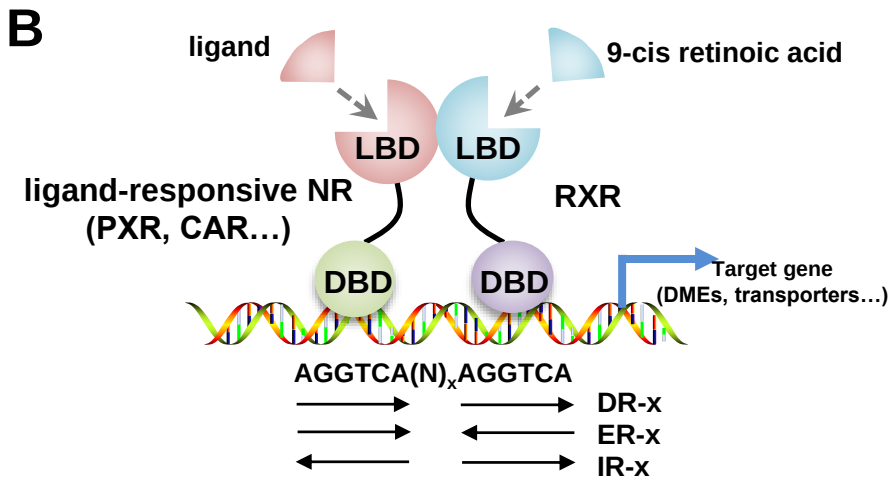


Figure 4





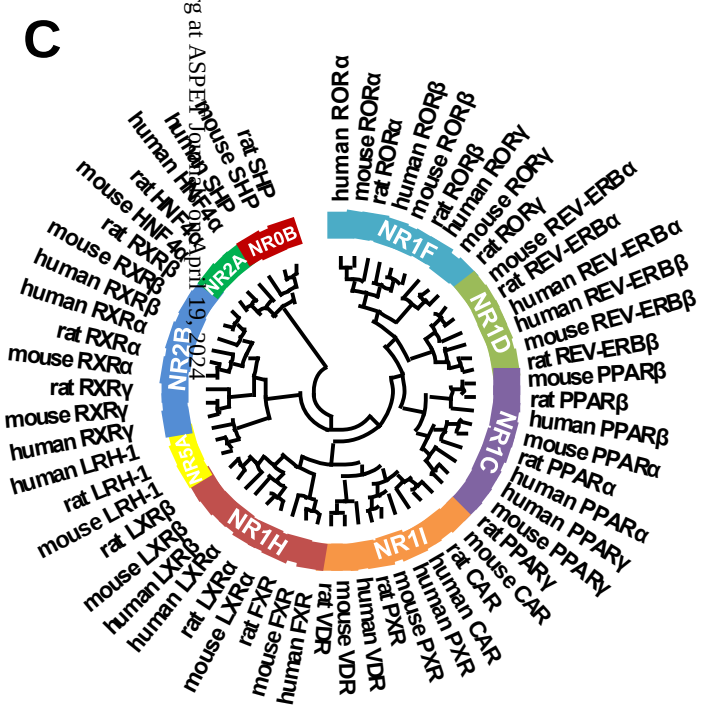


Figure 5

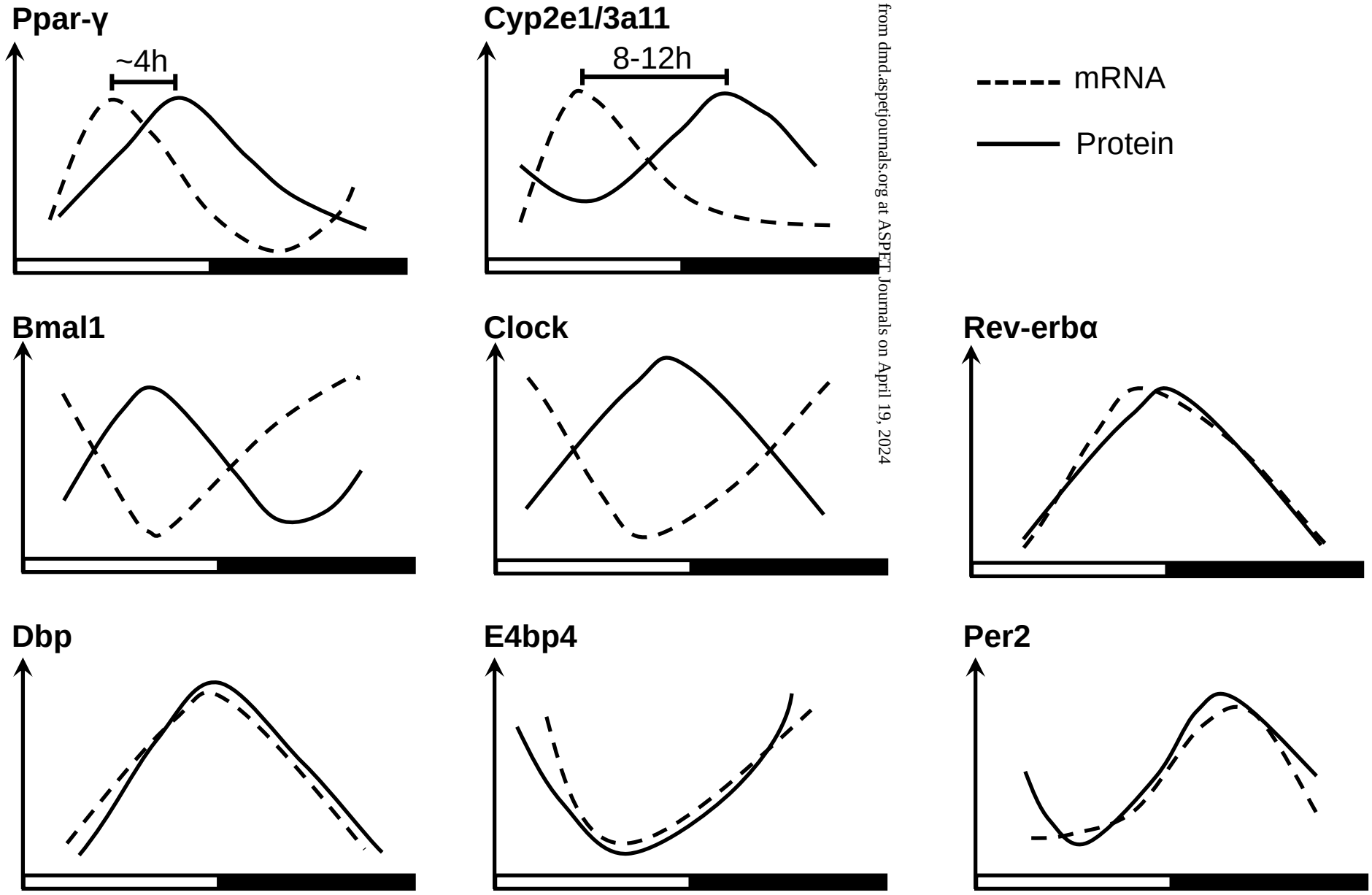


Figure 6

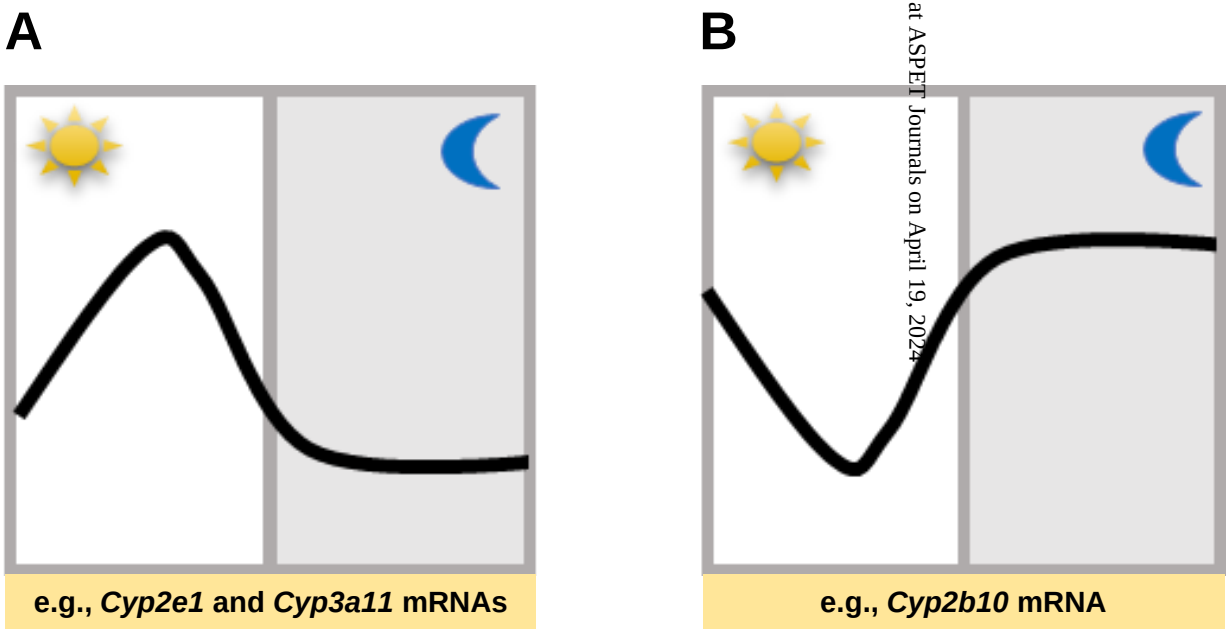


Figure 7

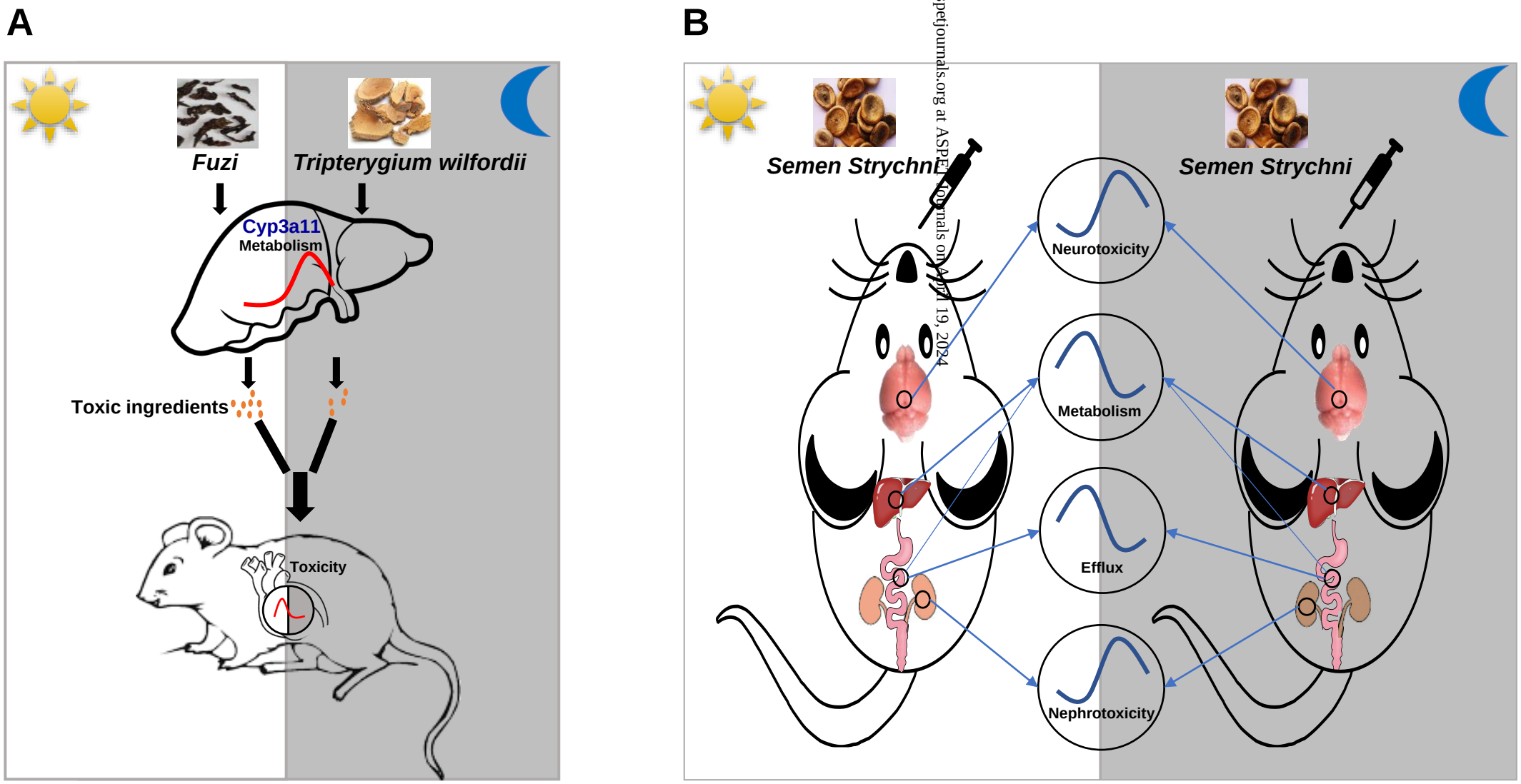


Figure 8

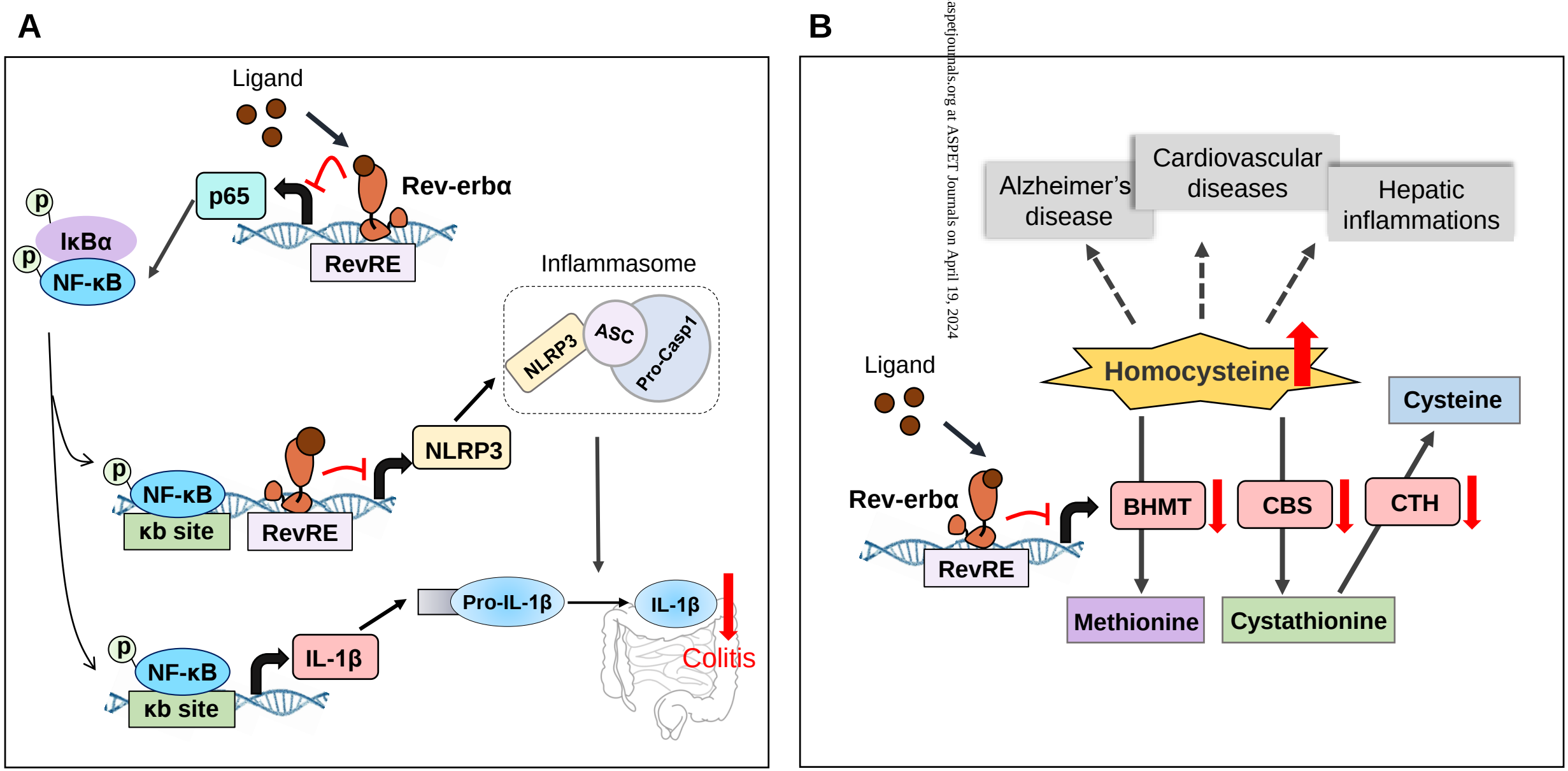


Figure 9