

# Circadian rhythms and new options for novel anticancer therapies

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**Abstract:** The patterns of activity/sleep, eating/fasting, etc show that our lives are under the control of an internal clock. Cancer is a systemic disease that affects sleep, feeding, and metabolism. All these processes are regulated by the circadian clock on the one hand, but on the other hand, they can serve as signals to tighten up the patient's circadian clock by robust daily routine. Usually, anticancer treatments take place in hospitals, where the patient's daily rest/activity pattern is changed. However, it has been shown that oncology patients with a disturbed circadian clock have poorer survival outcomes. The administration of different anticancer therapies can disturb the circadian cycle, but many cases show that circadian rhythms in tumors are deregulated per se. This fact can be used to plan anticancer therapies in such a manner that they will be most effective in antitumor action, but least toxic for the surrounding healthy tissue. Metabolic processes are highly regulated to prevent waste of energy and to ensure sufficient detoxification; as a consequence, xenobiotic metabolism is under tight circadian control. This gives the rationale for planning the administration of anticancer therapies in a chronomodulated manner. We review some of the potentially useful clinical praxes of anticancer therapies and discuss different possible approaches to be used in drug development and design in the future.

**Keywords:** circadian rhythms, cancer, chronotherapy, detoxification metabolism

## Introduction

The patterns of activity/sleep, eating/fasting, etc show that our lives are under the control of an internal clock. The major quality of the circadian clock is its maintenance in constant darkness (free-running conditions), while light at dawn serves as a resetting signal to set our clocks to approximately 24 hours.<sup>1,2</sup> This clock is in the hypothalamus of the brain in the suprachiasmatic nucleus (SCN), a region above the optical chiasm (reviewed by Okamura<sup>3</sup> and Weaver<sup>4</sup>). The SCN receives photic signals from ganglion cells in the retina, which enables the SCN to sense the start of each day at dawn. The clock in the SCN is the central oscillator, but many other tissues have their own clocks as well – peripheral oscillators that regulate different tissue-specific metabolic processes. These metabolic processes have to be tightly regulated to prevent waste of energy and to ensure sufficient detoxification. Circadian clocks are maintained with finely regulated molecular mechanisms ticking in each individual cell, different cells in a tissue need to be orchestrated, and clocks between different tissues need to be synchronized. Only in that way can an organism be fully functional. If desynchronization of any of these systems occurs, or if the circadian clock stops ticking because of mutations in clock genes, then different discordances can occur that lead to the development of different diseases, including cancer.

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## Molecular mechanisms of circadian rhythms

The clock responsible for circadian regulation in free-running conditions is represented by a set of transcription activators and repressors. Genes encoding the core clock components are transcribed periodically. They are involved in transcriptional–translational–post-translational modification loops that serve autoregulation as well as the regulation of circadian expression of output genes. The first core clock gene identified was Circadian locomotor output cycles kaput (*Clock*).<sup>5,6</sup> It is accompanied by transcription factors Brain and muscle Arnt-like protein-1 (*Bmal1*) and Neuronal PAS domain protein 2 (*Npas2*) as activators, and Period (*Per*) 1 and 2 and Cryptochrome (*Cry*) 1 and 2 as repressors. On the protein level, CLOCK/BMAL1 or NPAS2/BMAL complex binds to E-box DNA elements in promoter regions of different genes, among others, and also to promoter regions of repressors *Per1,2* and *Cry1,2*.<sup>5,7,8</sup> PER heterodimerizes with the CRY, and they bind to the BMAL1/CLOCK (NPAS2) complex and attenuate its activity. As a consequence, the transcription of *Per* genes is stopped, completing the first autoregulatory loop. When the pool of PER and CRY proteins is diminished owing to protein degradation, PER and CRY are removed from the BMAL1/CLOCK complex, and transcription is reactivated.<sup>9,10</sup> The degradation of PER and CRY is controlled through posttranslational mechanisms.<sup>11,12</sup> In addition to this core clock regulation, several oscillators help to maintain the robustness and precision.

## Circadian regulation of cell cycle check points and DNA repair

The circadian clock is a cell-autonomous and self-sustained oscillator with a period of about 24 hours. In growth conditions, successive divisions and progression through the cell cycle can also be considered as a periodic process.<sup>13</sup> The cell cycle duration in mammalian cells typically lasts on the order of 1 day.<sup>14</sup> Cell cycle states fluctuate with circadian time in different organisms: cyanobacteria,<sup>15</sup> fungi,<sup>16</sup> zebra fish,<sup>17</sup> and mammalian cells.<sup>18,19</sup> Mitotic indices are known to exhibit clock-dependent daily variations.<sup>18,20,21</sup> This has led to a model where the circadian clock may establish temporal windows in which certain cell cycle transitions are favored or suppressed – circadian gating of the cell cycle. Study in a regenerating mouse liver showed that WEE1, which limits the kinase activity of CDK1 and prevents entry into mitosis, is controlled at the transcription level with CLOCK/BMAL1 and shows circadian activity. So it is functioning as

a clock-dependent cell cycle gate.<sup>22</sup> In primary fibroblasts, protein NONO interacts with PER and gates S-phase to specific circadian times.<sup>19</sup>

The hallmark of cancer is disrupted control of the cell cycle. Normal cell cycle progression requires several control check points, and if these are deregulated, this could lead to cancerogenesis.<sup>23</sup> Many anticancer drugs act through the induction of DNA damage that causes irreparable lesions and stops the replication of DNA and leads to cell senescence. This effect is known as genotoxic stress.<sup>24</sup> Genotoxic treatments mainly target rapidly dividing cells: bone marrow, intestinal epithelium, and hair follicles, and therefore cause common side effects such as myelosuppression, mucositis, and alopecia. All these tissues harbor functional clocks, and the circadian regulation of cell cycle check points could help protect normal tissues from genotoxic stress-induced treatments.<sup>25,26</sup>

Sensing the DNA damage at the cell cycle check points is mediated by two protein kinases: ATM (ataxia telangiectasia mutated) and ATR (ATM-Rad3 related).<sup>27</sup> ATM is activated when DNA double-strand brakes occur and phosphorylates CHK2 kinase. PER1 interacts with ATM/CHK2 complex. When *Per1* is downregulated, ATM-dependent phosphorylation of CHK2 is impaired. Tumor cell lines with downregulated *Per1* are therefore more resistant to anticancer drugs.<sup>28</sup> TIM (*Drosophila* homolog TIMELESS) associates with core circadian proteins PER and CRY.<sup>29</sup> Human TIM interacts with cell cycle check point proteins CHK1, ATR, and ATR small subunit ATRIP. These interactions are stimulated with hydroxyurea and UV light, which cause DNA damages. Downregulation of TIM results in ATR-dependent phosphorylation of CHK1.<sup>30</sup>

If the damage of DNA does appear, the cell has different mechanisms to repair it. One of the mechanisms to repair single-strand DNA brakes is nucleotide excision repair (NER). CRY evolved from the family of cryptochrome/photolyases,<sup>31</sup> and this fact suggests that CRY could be involved in DNA repair mechanisms. Indeed, plant CDP-photolyases interact with the CLOCK/BMAL complex in a similar fashion as mammalian CRYs, and they are able to compensate for *Cry* deficiency and restore circadian oscillations in cell lines and liver.<sup>32</sup> Mammalian CRYs regulate the NER mechanism, which displays daily oscillations in the brain and liver.<sup>33,34</sup> NER also removes cisplatin-induced DNA damage, and this activity displays circadian oscillations in liver extracts.<sup>34</sup> NER is constantly high in *Cry*-deficient mice, which suggests that circadian clock downregulates the activity of NER at certain times of the day. Development of

skin tumors depends on time of exposure to carcinogens and negatively correlates with NER activity.<sup>35</sup>

The circadian clock mechanism is also involved in the double-strand breaks repair mechanism. When checked for genes that are involved in mitomycin C sensitivity, the list of candidate genes included also CLOCK protein. In experiments with laser-induced damage, CLOCK was shown to colocalize with  $\gamma$ -H2AX protein, marker of double-strand DNA break sites.<sup>36</sup> These findings define core circadian clock protein CLOCK as a regulator of several mechanisms of DNA repair induced by different genotoxic agents.<sup>37</sup>

## Circadian rhythms and cancer

The circadian clock regulates the normal cell cycle and apoptosis,<sup>38</sup> and anticancer drugs usually target different stages of the cell cycle, so differences in the cell cycle in cancerous versus healthy cells represent the rationale of anticancer chronotherapy.<sup>39</sup> In mice and rats, rhythm in tolerance of many anticancer drugs was shown: cytokines, cytostatics, antiangiogenic agents, cell cycle inhibitors, etc.<sup>39</sup> Experimental evidence shows that both dose and circadian timing play a critical role in antitumor efficacy, using tumor growth inhibition and increase in life span as measure.<sup>39</sup>

Alterations in clock genes in tumors versus healthy tissue can cause increased susceptibility to develop cancer and poor patient survival. This was shown for colorectal cancer,<sup>40,41</sup> chronic lymphocytic leukemia,<sup>42</sup> epithelial ovarian cancer,<sup>43</sup> and breast cancer.<sup>44</sup> In various epidemiological studies, it was shown that single nucleotide polymorphisms in clock genes are associated with higher cancer risk for prostate cancer (*CRY2* rs1401417: G>C, 1.7-fold higher risk),<sup>45</sup> breast cancer (*NPAS2* Ala394Thr),<sup>46</sup> non-Hodgkin's lymphoma (*CRY2* rs11038689, rs7123390, rs1401417),<sup>47</sup> and colorectal carcinoma (*CLOCK1* 311T>C, CC 2.78-fold and TC 1.78-fold higher risk).<sup>48</sup> Gene expression of all three *PERs* is deregulated in breast cancer cells, and *PER1* expression is downregulated in most patients.<sup>49</sup> *PER1* is downregulated in non-small lung cancer tissues compared with matched normal tissues.<sup>28</sup> *PER2* mRNA levels are downregulated in several human lymphoma cell lines and in acute myeloid leukemia patients.<sup>50</sup> In breast cancer, *PER2* can bind to and destabilize estrogen receptor  $\alpha$ .<sup>51</sup> Polymorphisms in the number of tandem repeats in the polymorphic domain of *PER3* can cause circadian disruption, increase inflammation by increased IL-6 levels, and therefore increase cancer risk.<sup>52</sup> Mutations in *NPAS2* are associated with increased risk for breast cancer and non-Hodgkin's lymphoma.<sup>47</sup>

*Per2* mutant mice are predisposed to oncogenic transformation following  $\gamma$ -radiation,<sup>53</sup> and crossing of *Per2* mutant mice with *Apc*<sup>Min/+</sup> increases the frequency of polyp formation.<sup>54,55</sup> Downregulation of *Per2* expression increases tumor growth in vivo,<sup>56</sup> and overexpression inhibits tumor growth.<sup>57</sup> Similar effects are also observed for *Per1*.<sup>28,58</sup> Lower levels of *Per1* and *Per2* expression are found in human colorectal cancer tumors.<sup>59,60</sup> All of the foregoing show that functional circadian clock might operate as a tumor suppressor.<sup>61</sup> Further evidence that circadian clock is disrupted in cancer is shown in the study of Soták et al:<sup>61</sup> the expression profile of *Dbp* had robust circadian rhythmicity in the colonic mucosa of healthy mice and in the surrounding nonneoplastic tissue of tumor-bearing mice, but the amplitude of the tumors was reduced, and the amplitude was delayed. The rhythmic expression of core clock genes was markedly reduced in comparison with surrounding nonneoplastic tissue, and even more in comparison with colon mucosa of healthy animals.

Animal models used for studying tumors were treated with azoxymethane (AOM), following consumption of dextrane sodium sulfate (DSS). Soták et al<sup>61</sup> showed that circadian rhythm in AOM mice changed, the possible mechanisms being: mutations of K-ras cascade lead to activated MAPK/ERK pathway, which represses BMAL1 by phosphorylation by MAPK; mutations in  $\beta$ -catenin activate the *Wnt* signaling pathway and prevent GSK3 $\beta$  phosphorylation that can modulate *PER2*<sup>55</sup> and BMAL1<sup>62</sup> protein stability; mutations in the TGF $\beta$  pathway cause TGF $\beta$  to bind to its type 2 receptor, as a result of which phospho-SMAD3 forms a dimer with SMAD4 and activates the transcription of the *Dec1* gene – regulator of the clock.<sup>63</sup> All these pathways can also be involved in the induction of tumorigenesis.

A recent article describes using the cell line systems where RAS mutations were induced and transcription was measured with microarrays according to the circadian time. Results indicate that this mutation, which is a common driver of cancers (especially colorectal cancer), deregulates the circadian clock in cancers.<sup>64</sup> These results raise the question as to whether the deregulation and desynchronization of the circadian clock (as in shift-work)<sup>65</sup> cause cancer or whether cancer-causing mutations alter the circadian clock in tumors, thereby causing them to desynchronize.<sup>64</sup>

## Epigenetic mechanisms and circadian clock

Epigenetic mechanisms are modifications of histones and/or methylation of DNA that influence compaction of chromatin

and accessibility of gene promoters to the transcription machinery. They are considered as mechanisms of transcription regulation.<sup>66</sup> Genes encoding circadian clock proteins are regulated by epigenetic mechanisms, such as histone phosphorylation, acetylation, and methylation, which have been shown to follow circadian rhythm.<sup>67,68</sup> CLOCK/BMAL1-mediated activation of clock-controlled genes has been shown to be accompanied by circadian changes in histone acetylation at their promoters.<sup>69</sup> In fact, the transcriptional factor CLOCK has also histone acetyltransferase activity.<sup>70</sup> CLOCK binds to E-box regions of DNA and can activate the transcription of clock-controlled genes. CLOCK can also acetylate nonhistone proteins: it acetylates its transcription partner BMAL1 and facilitates CRY-dependent repression.<sup>71</sup> CLOCK associates also with histone methyltransferase MLL1 (mixed lineage leukemia 1) and recruits it to the promoters of clock-controlled genes.<sup>72</sup> MLL1 methylates histone H3 at lysine 4 (H3K4). This modification at the promoters of clock-controlled genes defines the active chromatin state and is rhythmical.

Histone deacetylase SIRT1 is a modulator of circadian clock machinery.<sup>73</sup> SIRT1 activity is NAD<sup>+</sup> dependent and is therefore metabolically regulated.<sup>74</sup> The activity of SIRT1 is circadian and is directly driven with rhythmic oscillations in NAD<sup>+</sup> levels.<sup>75</sup> The CLOCK/BMAL1 complex interacts with SIRT1 and recruits it to the promoters of rhythmic genes. SIRT1 also deacetylates nonhistone proteins such as BMAL1 (signal for CRY recruitment)<sup>71</sup> and PER2 (enhances stability).<sup>73</sup> SIRT1 also regulates several proteins involved in metabolism and cell proliferation. Gluconeogenesis is regulated via deacetylating PPAR $\gamma$ -coactivator  $\alpha$  (PGC1 $\alpha$ ) and Forkhead box O1 (FOXO1).<sup>76</sup> FOXO1 directly regulates several gluconeogenic genes and PGC1 $\alpha$  coactivates glucocorticoid receptor (GR). SIRT1 also regulates Liver X receptor (LXR) that regulates cholesterol metabolism.<sup>76</sup>

Histone deacetylase 3 (HDAC3) modulates acetylation of circadian genes responsible for lipid metabolism. REV-ERB $\alpha$  is controlled by the nuclear receptor corepressor 1 (NCoR1), which recruits HDAC3 to mediate transcriptional repression of target genes, such as *Bmal1*. HDAC3 recruitment to the genome was recently shown to be rhythmic in liver, and the depletion of either HDAC3 or REV-ERB $\alpha$  was shown to cause fatty liver phenotype.<sup>77</sup> HDAC1 forms a complex with PER2, is recruited to the *Per1* promoter, and represses its transcription.<sup>78</sup>

Altered epigenetic marks of different genes are described in several cancers regardless of anatomic region.<sup>79,80</sup> Some of them are considered as possible diagnostic and prognostic

cancer markers.<sup>81</sup> DNA methylation is especially interesting because of its stability and simple accessibility in routine molecular diagnostic laboratories. Several regions of DNA are being considered for DNA methylation measurements also for the purposes of cancer screening programs.<sup>82,83</sup>

Acetylation-mediated epigenetic regulation of GR activity is important in the context of circadian regulation.<sup>84</sup> The HPA axis (hypothalamic–pituitary–adrenal) is important not just as a stressor regulating system, but also as the most likely mediator of circadian signals from the central system in the SCN to the peripheral organs. Hormones involved in the HPA axis regulate one another's secretions: corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) stimulate the pituitary gland to secrete adrenocorticotrophic hormone (ACTH), which in turn stimulates the adrenal gland to produce glucocorticoids that are secreted in a circadian manner. Secreted glucocorticoids suppress higher regulatory centers, the hypothalamic paraventricular nucleus and the pituitary gland, forming a closed negative feedback loop that resets the activated HPA axis and restores its homeostasis.<sup>85</sup> The circulating levels of corticosteroids are circadian. In humans, cortisol zenith is reached in the early morning, and the nadir at midnight.<sup>85</sup>

Glucocorticoids function is regulated with the expression of the GR (nuclear receptor superfamily 3, group C, member 1 – NR3C1). It is expressed virtually in all organs and tissues of the human body. GR is present in the cytoplasm bound to a larger protein complex; after binding glucocorticoids, it is conformationally changed and translocated to the nucleus, where it binds to a specific glucocorticoid response element (GRE), in the promoter regions of various genes. GR can act as an activator or repressor regarding the coregulators that bind to it.<sup>85</sup> Human GR can be acetylated, and acetylation of GR attenuates the repressive effect of GR on nuclear factor  $\kappa$ B.<sup>86</sup> CLOCK acetylates GR in the nucleus, after GR binds the glucocorticoids in the cytoplasm and has translocated to the nucleus.<sup>87</sup> The rhythm of peripheral CLOCK activity (including acetylation) is circadian, and therefore the activity of GR is circadian as well and is opposite that of maximal glucocorticoid levels in the blood.<sup>88</sup> If this fine-tuned system is deregulated, it may lead to functional hypercortisolism in target tissues, which could be associated with development of pathologic conditions.<sup>88</sup> Glucocorticoids regulate the immune response, and deregulation could lead to chronic inflammation, which is a common physiological state of cancer. If the theory of cancer immunosurveillance and immunoediting is considered, a deregulated and dysfunctional immune system could speed up the escape phase

of tumor cells that would otherwise be eliminated by the immune system.<sup>89</sup>

Recently, it was shown that histone deacetylase SIRT1 can directly regulate the expression of *BRCA1*, the gene, if mutated, that is associated with hereditary familial breast and ovarian cancers.<sup>90</sup> *BRCA1* inactivation events (mutation, promoter methylation, or knockdown) were accompanied by decreased SIRT1 levels and increased NAD<sup>+</sup> levels, and a subsequent increase in SIRT1 activity. It is yet to be proved whether the *BRCA1* mutation carriers show deregulated circadian clock in organs where *BRCA1* seems to be of extreme importance (breast and ovary).

## Circadian regulation of drug metabolism

For the purposes of anticancer therapy design, it is essential to know how drug metabolism is regulated, at which time points according to circadian rhythm detoxification enzymes are most effective, and when clearance of drugs will be the highest.

### General

Nuclear receptors are sensors for induction of proper detoxification enzymes. They are expressed in liver, white and brown adipose tissue, and display rhythmic patterns of expression.<sup>91</sup> From the group of nuclear receptors involved in xenobiotic metabolism, the pregnane X receptor (PXR), the constitutive androstane receptor (CAR), and the small heterodimer partner (SHP) are strongly oscillating in the liver.<sup>91</sup> The three PAR bZIP proteins DBP, TEF, and HLF are direct output mediators of the core circadian clock and are expressed in the liver where they regulate various genes involved in detoxification and drug metabolism. Triple knockout mice of PAR bZIP proteins (DBP, TEF, HLF) show a high morbidity rate and hypersensitivity to xenobiotic compounds and absence of CAR circadian expression profile.<sup>92</sup>

Nuclear receptors upregulate the expression of xenobiotic metabolizing phase I enzymes: CYPs, alcohol dehydrogenases, aldehyde dehydrogenases (*Aldhs*), carboxylesterases (*Cess*), and paraoxonases (*Pons*). They have oxidase, reductase, or hydroxylase activities<sup>93</sup> and are regulated with ALAS1 and P450 oxidoreductase (POR). All CYPs require heme as a prosthetic group, and ALAS1 is the rate-limiting enzyme in heme biosynthesis.<sup>94</sup> The availability of heme is strongly circadian, and so is the expression of *Alas1*, which is regulated by NPAS2.<sup>95</sup> The transcription of both *ALAS1* and *POR* is decreased in PAR bZIP triple knockout mice.<sup>92</sup> In these mice, the expression pattern of phase I enzymes (*Cyp2b*, *Cyp2c*, *Cyp2a*, and *Cyp3a*) is also changed: for *Cyp3a4*,

*Cyp2a4*, and *Cyp2a5*, direct binding of DBP was shown.<sup>96,97</sup> The phase II group are conjugating enzymes, and they consist of many superfamilies of enzymes: sulfotransferases (SULT), UDP-glucuronosyltransferases (UGT), NAD(P)H-quinine oxidoreductases (NQO), epoxide hydrolases (EPH), Glutathione S-transferases (GST), and N-acetyltransferases (NAT).<sup>98</sup> Most phase II enzymes show diurnal variation in expression. Phase II conjugation of xenobiotics in liver has a circadian rhythm with more glutathione conjugation in the early light phase, glucuronidation in the late light phase, and sulfation in the early dark phase.<sup>93</sup> In the group of phase III metabolizing genes, we find transporters that help in the uptake of xenobiotics from the blood to the liver or in the elimination of the metabolized xenobiotics, and many of them show circadian rhythmicity in expression.<sup>99</sup> Most of these phase III enzymes were initially described as chemoresistance proteins overexpressed in cancer cell lines. It may be interesting to investigate whether these transporters display a diurnal rhythmicity in visceral cancers in vivo, as this would have strong therapeutic implications.<sup>100</sup>

## Circadian regulation of anticancer drugs metabolism

Circadian timing system also regulates metabolism of most anticancer drugs. The circadian rhythm of seliciclib, docetaxel, irinotecan, mitoxantrone, and vinorelbine is probably the consequence of the circadian rhythm of *Cyp3a*.<sup>101,102</sup> Circadian tolerability of cyclophosphamide is regulated by the rhythmic *Cyp2b10* and possibly *Cyp2c29*. Clock-control transcription factors *Dbp*, *Tef*, and *Hlf* regulate the expression of *Cess1* and *Cess2*, which account for the increased biotransformation of irinotecan to SN-38 during the light–rest phase of mice.<sup>101,103</sup> All enzymatic activities that generate the cytotoxic forms of 5-FU are highest during the dark–active phase in mice, when 5-FU is most toxic to healthy tissues.<sup>104</sup> The toxicity of platinum complexes is determined by the rhythmic phase of reduced glutathione (GSH). Liver and jejunum GSH levels are ten fold higher in the second half of the night compared with midnight in mice.<sup>105,106</sup> UGT1A catalyzes detoxification of seliciclib, irinotecan, and SN-38. The highest UGT activity is reported during the dark–active phase of rats.<sup>107</sup> But no consistent relationship is found between blood chronopharmacokinetics and chronotolerance for irinotecan, cyclophosphamide, cisplatin, carboplatin, oxaliplatin, interferon  $\beta$ , or seliciclib.<sup>39</sup> The highest elimination is observed in mice treated with carboplatin at ZT8 and with oxaliplatin at ZT16, despite both drugs being least toxic at ZT16.<sup>108</sup>

## Chronomodulating drugs and their potential in cancer treatment

One of the attractive ideas is to use chronomodulating drugs that could reset or hold the circadian cycle on a specific time point to be able to treat patients when the treatment would be most effective or help to restore the dampened clock because of the disease itself or the application of the treatment.

Circadian oscillation in cultured cells can be induced by different agents: high serum concentration,<sup>109</sup> dexamethasone (GR agonist),<sup>110</sup> forskolin (activator of adenylate cyclase),<sup>111</sup> phorbol-12-myristate-13-acetate (PMA, activator of protein kinase C), fibroblast growth factor (FGF), epidermal growth factor (EGF), insulin, calcium ionophore calcimycin (induces apoptosis by intracellular  $Ca^{2+}$ ),<sup>112</sup> endothelin,<sup>113</sup> glucose,<sup>114</sup> prostaglandin E<sub>2</sub>,<sup>115</sup> NAD<sup>75,116</sup>, heme,<sup>117</sup> and cAMP.<sup>118</sup> These compounds induce rhythm through different pathways.<sup>119</sup> Thus, many different compounds could be used to modulate the circadian clock on different points of the circadian cycle.

One of the experimental approaches is to screen different chemicals and observe their effect on the circadian cycle with the use of stably transfected Luc reporter cell lines. One of such screens revealed the drug that inhibits glycogen synthase kinase 3 $\beta$  (GSK-3 $\beta$ ), which shortens the period of oscillations in U2OS cells.<sup>120</sup> In mammals, GSK-3 $\beta$  has been previously identified as a kinase that directly phosphorylates several core clock proteins and mediates their degradation (CRY2,<sup>121</sup> CLOCK,<sup>122</sup> and BMAL1<sup>62,123</sup>); increases nuclear translocation (PER2<sup>122</sup>); or increases stabilization (REV-ERB $\alpha$ <sup>124</sup>). Another group identified a small molecule, named longday-sin, that lengthens the circadian period of different cultured cells as well as mouse SCN explants.<sup>125</sup> Longdaysin targets several protein kinases, CK1 $\delta$ , CKI $\alpha$ , and ERK2. The role of CK1 $\delta$  and ERK2 in the regulation of the circadian clock was previously known, but CKI $\alpha$  was new and it appeared that it directly phosphorylates PER1 and promotes its degradation.<sup>125</sup> If other parameters, such as amplitude of oscillation or rhythmicity, are considered, a wide screen identified several small molecules, that caused a significant increase in the amplitude.<sup>126</sup> That correlates with the expression of clock-output genes such as *Dbp* and *Rev-Erb $\alpha$* . Some of the newly identified small molecules mediated acute induction of *Per2*, followed by the phase delay, as seen in the effect of forskolin on SCN slices.<sup>118</sup> Another screen identified a small molecule that prevents degradation of CRY and lengthens the circadian period.<sup>127</sup> This allows studies of gluconeogenesis, since CRY negatively regulates the transcription of two rate-limiting enzymes, phosphoenolpyruvate carboxykinase 1

(*Pck1*) and Glucose-6-phosphatase (*G6pc*).<sup>128,129</sup> Treatment with this compound repressed glucagon-mediated induction of *Pck1* and *G6pc* and production of glucose, so it could be considered a potential clock-based therapy for treatment of diabetes.<sup>127</sup>

Many core clock proteins have also clock-independent physiological functions,<sup>130</sup> so small molecules that would modulate individual clock proteins may be considered as more specific therapeutic drug. CLOCK/BMAL1 functionality was changed during genotoxic treatment,<sup>102</sup> so screen for modulators of its functionality was performed.<sup>131</sup> These studies used mice with different impaired clock genes, and although all were behaviorally arrhythmic, they displayed an opposite response to toxicity induced by chemotherapeutic cyclophosphamide. Animals with a deficiency of clock activators (*Clock* mutant mice, *Bmal1* KO mice) were extremely sensitive to cyclophosphamide, whereas mice with a deficiency of clock repressors (*Cry* double KO mice) were resistant to the treatment. This data suggests that circadian transcriptional activators could be potential targets for pharmacological modulation to protect normal tissues from damage induced by genotoxic treatments. A screen of CLOCK/BMAL1 activity modulators revealed several known regulators of circadian function<sup>132</sup> as well as some new chemicals such as organic selenium compound L-methyl selenocysteine.<sup>131</sup> Selenium prevents the binding of transcription repressor *Tiegl* to the SP-1 binding site in *Bmal1* promoter, upregulates *Bmal1* transcription, increases BMAL1 protein and probably activates the CLOCK/BMAL1 complex. This effect was shown in vitro as well as in vivo with the use of a selenium-supplemented diet or by injecting it. The in vivo effect was tissue-specific, since selenium-induced BMAL1 effects were seen in the liver, but not in the SCN. This means that there was no change in the behavioral parameters. This presents huge therapeutic potential, since it does not disturb the central clock. This mechanism is mediated through BMAL1 only, since selenium failed to reproduce the effects in *Bmal1* KO animals. Selenium already has two major clinical implications: tumor prevention and protection against DNA damage induced by anticancer therapy – radiation. Selenium supplementation moderates mucositis induced with fractionated doses of ionizing radiation<sup>133</sup> as well as diarrhea in treated patients with cervical and uterine cancers.<sup>134</sup>

CLOCK/BMAL1 functionality was also studied in the *Cry*-deficient mouse model, where an interesting mechanism of pharmacological interest was described. It has been found that *Cry* double KO mice in *p53*-null background are rendered

more sensitive to UV-light-induced apoptosis.<sup>135</sup> This increase was due to CLOCK/BMAL1 upregulation of *p73*-dependent apoptosis. In the absence of *p53*, downregulation of *Cry* enhances the expression of *p73*, and this correlates with increased levels of the *Egr1* (early growth response 1) gene. *Egr1* is a positive activator of *p73*<sup>136</sup> and is itself directly regulated by CLOCK/BMAL1. *Egr1* is consistently upregulated in *Cry*-deficient cells owing to downregulation of BMAL1.<sup>137</sup> EGR1 binds to the promoter of *p73* and is a positive regulator. Negative regulator C-EBP $\alpha$  is also present at the *p73* promoter, but upon exposure to UV light, only EGR1 stays bound to it. A similar effect was observed when tumor xenografts were treated with oxaliplatin.<sup>137</sup> These findings suggest a therapeutically interesting mechanism for sensitizing tumor cells that are deficient in *p53* function through the activation of the *p73*-dependent apoptotic pathway.

## Chronotherapies in oncology: possibilities and options

The idea of chronotherapy is to administer each drug according to a delivery pattern with precise circadian times in order to achieve the best tolerability and efficacy.<sup>138</sup> Multichannel programmable pumps enable drug administration according to precisely timed infusion rates in order to deliver chronotherapy with minimal interference with the circadian pattern of the daily life of the patient.<sup>139</sup> Even oral chemotherapy can be administered in optimal chronotherapy fashion. The examples of good practices of oral chronotherapy are described for busulfan,<sup>140</sup> 6-mercaptopurine,<sup>141</sup> and oral fluoropyrimidines.<sup>142</sup> In future, oral chemotherapeutics could be delivered with chronoprogrammed release formulation that would not disturb the patient's nighttime sleep rhythm.<sup>143</sup>

Oncological chronotherapies were extensively studied on rodents. Circadian timing largely modifies the extent of toxicity of many anticancer drugs: cytostatics, cytokines, and targeted biological agents. A lethal dose of any of these drugs results in two fold to more than ten fold changes as a function of circadian timing of drug administration.<sup>39</sup> Such large differences occur irrespective of delivery route (oral, intravenous, intraperitoneal, or intra-arterial) or the number of daily or weekly administrations. Circadian rhythms in the tolerability of anticancer drugs persist in rodents kept in constant darkness or in constant light, which demonstrates their endogenicity. Circadian time for best tolerability and efficacy was determined for several chemotherapeutic drugs, and both times overlap. 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,<sup>144</sup> irinotecan–oxaliplatin,<sup>145</sup> or gemcitabine–cisplatin<sup>146</sup> in B6D2F1 mice, and docetaxel–doxorubicin in C3H/He mice.<sup>147</sup>

The first clinical study that showed that therapy timing is important for the best outcome was on patients with non-small cell lung cancer.<sup>148</sup> Trials with ovarian cancer patients showed better tolerability as compared with treatment 12 hours apart. DNA-intercalating agents doxorubicin and epirubicin were best tolerated when administered in the morning, and alkylating-like drug cisplatin when administered in the late evening.<sup>149,150</sup> This initial finding did not alter the clinical practice for drug administration because of practical difficulties until the development of programmable intravenous systems that enable chronomodulated administration of up to four drugs. A good example is commonly used chemotherapeutic oxaliplatin that was initially found too toxic in Phase I clinical testing for use in colorectal carcinoma. Chronotherapeutic mouse studies revealed a ten fold change in toxicity with respect to dosing time.<sup>108</sup> This finding led to the randomized Phase I two-arm study: in the first one, patients received chronomodulated infusion with peak at 4 pm as compared with the second arm, where patients were treated with constant rate infusion. Chronotherapy induced fewer peripheral sensory neuropathies, as this is the most common side effect of the drug.<sup>151</sup> A study on metastatic colorectal cancer patients showed that most antitumor activities were recorded in the group of patients on chronotherapy.<sup>152</sup>

Chronomodulated oxaliplatin infusion was combined with 5-fluorouracil-leucovorin (5-FU-LV) with a peak flow rate at 4 am. On the incidence of mucosal toxicities and the peripheral sensory neuropathy, the researchers concluded better tolerance as compared with constant rate infusion.<sup>153,154</sup> The best tolerated chronotherapy schedule also achieved best tumor shrinkage.<sup>24</sup> When compared with the conventional delivery schedule of the same drugs, overall survival was similar in both treatment groups.<sup>155</sup> Chronomodulated drug delivery significantly reduced the risk of early death in male patients by 25%, while the opposite finding was recorded for female patients. Median survival showed a difference of 6 months between men and women on the chronomodulated drug delivery regime, while no sex-related difference in survival was found in patients on a conventional delivery regime. The majority of preclinical studies were performed on male mice, and findings adequately predict the optimal timing for male patients, since the researchers did not have any valid prediction for female patients. It has been shown that sex difference is important in chronotolerance of irinotecan in

mice.<sup>156,157</sup> The rhythmic expression of almost 2,000 genes when compared in the oral mucosa of healthy male and female volunteers differed. In this group, we can also find clock-controlled genes important for drug metabolism and cellular proliferation.<sup>20</sup> In general, drug metabolism pathways indeed display sex differences.<sup>158</sup> *Cry1* and *Cry2* seem to be important to sustain sex-related differences in drug metabolizing cytochrome P450 dimorphism.<sup>159</sup>

In the conventional delivery protocol, the higher the toxicity is, the more efficient is the chemotherapy, but in the chronomodulated delivery protocol, the higher the toxicity, the poorer the outcome predicted. Toxicity was measured with the rate of severe neutropenia.<sup>160</sup> The European clinical trial confirmed that chronomodulated therapy of hepatic metastasis of colorectal cancer with irinotecan, 5-FU, and oxaliplatin administered through hepatic arterial infusion was safe and that one-third of patients manifested tumor shrinkage.<sup>161</sup> Design of the time schedule proved to be important also for radiation therapy in patients with head and neck cancer, where morning procedures caused less severe oral mucositis as compared with afternoon procedures.<sup>162</sup> Similarly, the morning gamma knife radiosurgery doubled median survival as compared with the afternoon procedure.<sup>163</sup>

Another approach for anticancer treatment is immunotherapy, where therapies are focused on the adaptive immune response system: on cytotoxic T cell response and, more recently, stimulation of CD4+ T helper cells (Th).<sup>164</sup> Th cells activate antigen-specific effector cells and recruit the innate immune system such as macrophages, eosinophil granulocytes, and mast cells. Tumor antigen-specific Th cells are activated by either antigen-presenting cells or directly by major histocompatibility complex (MHC) class II expressing tumors. Many vaccine strategies aim to stimulate the Th response specific for a tumor antigen. Immune functions show circadian variations: circulating antibodies, total lymphocytes, and cell-mediated immune responses.<sup>165</sup> Circadian rhythm thereby influences the organization of cellular immune function. Levels of T cytotoxic lymphocytes, natural killer cells, and  $\gamma\delta$ TCR-bearing cells in peripheral blood, show the lowest levels at night and rise to a maximum around midday, whereas CD4+ Th cells have higher nocturnal levels.<sup>165,166</sup> In addition, circadian changes can be observed in the process of cell production, release and action of cytokines and chemokines that influence cell redistribution to the bone marrow, mobilization, and migration to lymphoid and nonlymphoid organs. Different lymphocytes populations, the level of IL-2, melatonin, and cortisol were compared in

normal healthy controls with non-small lung cancer patients. In healthy controls, circadian rhythm was observed in CD8+, CD16+,  $\gamma\delta$ TCR cells, and cortisol levels peaking at daytime, and in CD3+, CD4+, CD20+ cells, and melatonin levels peaking at nighttime. In non-small lung cancer patients, circadian rhythm was observed in CD16+,  $\gamma\delta$ TCR cells, and cortisol levels peaking at daytime, and CD4+, CD25+ cells, and melatonin levels peaking at nighttime. Although levels differ, the similarity in peak times for some lymphocyte subsets and hormones suggests that timed circadian administration of immunotherapy may improve the efficacy of treatment.<sup>165</sup> As evidence for this, it was shown that CD4+ T cell responses are regulated by a cellular circadian oscillator capable of driving rhythmic CD4+ T cell immune responses.<sup>167</sup>

Each person has his or her own circadian rhythm, and unlike studies performed on rodents with identical genetic background, these interindividual differences in humans should be taken into account when chronotherapies are modeled and designed. Depending on our everyday routine, we are ranked into different chronotypes that are dependent on age, sex, geographical location, and genotype.<sup>168</sup> To obtain informative data that are feasible in clinical settings, different noninvasive approaches are considered. One such measurement is actimetry, which is based on rest–activity pattern monitoring of the individual. To get reliable data, recording over several days (eg, 1 week) and the use of a wristwatch accelerometer are recommended.<sup>169</sup> Rest–activity pattern can differ largely among cancer patients.<sup>170,171</sup> Circadian rhythm disruption is a robust predictor of long-term survival outcomes in metastatic colorectal patients.<sup>170</sup> It was shown that chemotherapy could disrupt a patient's circadian rhythm, which is also a poor predictor of survival.<sup>160</sup> Another possible measurement of circadian rhythm is body temperature. The core body temperature can be measured with the use of rectal probes where high values usually occur in the late afternoon and the nadir is reached at late night.<sup>172</sup> This system is not particularly comfortable for cancer patients. The temperature can also be measured at skin surface: the highest measurement is usually recorded at early night, and the lowest in the early morning.<sup>173</sup> The measurement of skin surface temperature, the rest–activity pattern, and position recording was called TAP (Temperature-Activity-Position). TAP displayed stable measurement of individual rhythm compared with individual measurement and could be best served as a readout of cancer patient chronotype measurement.<sup>174</sup>

Patterns of hormones cortisol and melatonin vary during the day and can be measured noninvasively: in saliva



(cortisol and melatonin) and in urine (melatonin). Melatonin secretion usually peaks at early night and is strongly inhibited by night light in humans,<sup>175</sup> while cortisol secretion peaks around the waking hours, with lowest levels at early night.<sup>176</sup> The combination of these two can represent a new readout of the patient's chronotype. Salivary cortisol patterns are prognostic factors for the survival of metastatic breast cancer patients,<sup>177,178</sup> ovarian carcinoma patients,<sup>179</sup> and lung cancer patients.<sup>180</sup> No such relation was found for metastatic colorectal patients.<sup>181</sup>

## Conclusion

Experimental and clinical reports show that a robust circadian system is relevant for host control of cancer progression and treatment tolerability, that its disruption accelerates cancer progression, and that it is an independent prognostic factor for the survival of patients with different cancers.<sup>170,178,182</sup> Chronobiotics such as bright light, physical and social activity, meal timing, and sleep patterns could further strengthen or resynchronize the circadian timing system<sup>183,184</sup> and help to improve anticancer therapy outcome.

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## Disclosure

The author reports no conflicts of interest in this work.

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