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# Clocking cancer: the circadian clock as a target in cancer therapy.

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## Abstract

Disruption of the cellular pathway modulating endogenous 24-hour rhythms, referred to as 'the circadian clock', has been recently proven to be associated with cancer risk, development and progression. This pathway operates through a complex network of transcription-translation feedback loops generated by a set of interplaying proteins. The expression of core circadian clock genes is frequently dysregulated in human tumors; however, the specific effects and underlying mechanisms seem to vary depending on the cancer types and are not fully understood. Additionally, specific oncogenes may differentially induce the dysregulation of the circadian clock in tumors. Pharmacological modulation of clock components has been shown to result in specific lethality in certain types of cancer cells, and thus holds great promise as a novel anti-cancer therapeutic approach. Here we present an overview of the rationale and current evidence for targeting the clock in cancer treatment.

### Keywords

Clock genes; circadian rhythm; cancer; targeted therapy; CLOCK; BMAL1; REV-ERB; CRY; PER

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## INTRODUCTION

The circadian clock consists of a hierarchical multi-oscillator network of intracellular and intercellular mechanisms throughout the body that contributes to anticipating metabolic activity and maintaining system homeostasis in response to environmental cues and intrinsic stimuli. Any biological process that has roughly a 24-hour pattern can be said to have a circadian rhythm. Although most, perhaps all, organisms display circadian rhythms in their respective biological processes, circadian rhythms in mammals help optimize the transition between day and night for different behavioral and physiological activities (1). The circadian clock is a highly conserved self-sustained mechanism that regulates and synchronizes a wide variety of functions including sleep-wake cycles, rhythmical fluctuations of blood pressure, heart rate, body temperature and hormone secretion, and feeding behavior (2). The clock molecular mechanism is operated at the single cell level through a network of transcription-translation feedback loops (TTFLs) generated by a set of interplaying clock proteins, which regulate according to a circadian rhythm the expression of specific target genes and their products. Up to 20% of genes expressed in any particular cell or tissue have been found to undergo circadian oscillations at the mRNA level, indicating the extensive role of circadian gene regulation (3). As the genes showing circadian rhythmicity in different tissues are non-overlapping, more than 50% of genes in the body may be clock-regulated.

The disruption of the circadian rhythms in humans, resulting from sleep deprivation, jet-lag, shiftwork involving nightshifts, or unnatural light exposure, has been shown to have a deep impact on many physiological functions and has been related to several disorders including insomnia, depression, metabolic and cardiovascular diseases, and cancer. This can be explained by the clock-dependent modulation of several key cellular pathways regulating proliferation and apoptosis, DNA damage repair, cellular senescence, metabolic homeostasis, xenobiotic detoxification, oxidative stress, inflammatory and immune response (4). Over the past few years, genetic variations of core clock genes have been associated with cancer risk in several epidemiological studies and germline polymorphisms in these genes have been proposed as biomarkers of cancer risk, although genetic associations are not always consistent across different studies (5). This may partly be explained by the fact that the effect of gene variants on protein expression levels or function is often unknown. Mutations or loss of function of different clock proteins has, however, been confirmed in animal models to correlate with different pathological phenotypes and cancer risk.

Chronochemotherapy, as a way to apply chemotherapy against malignant tumors on a circadian schedule in order to optimize treatment efficacy and reduce toxicities by exploiting the physiology of cellular circadian rhythms, has been explored in several studies. Clinical trials of chronochemotherapy continue to be an active area of research, but have not consistently resulted in improved treatment efficacy to date (6). More recently, growing evidence suggests that small molecule drugs targeting the core components of the circadian machinery have promising anti-tumor activities. As such, circadian clock components are emerging as novel intriguing therapeutic targets for cancer treatment. Here we present an overview of the current evidence supporting targeting the clock as an actionable anti-cancer strategy.

# THE MAMMALIAN CIRCADIAN CLOCK

## Signaling to the Circadian Network

"Zeitgebers" or "time-givers" are any natural cue in the environment that can entrain an organism's biological clock. Zeitgebers include: light and dark cycle, food and drink, exercise, rest, social interactions, and temperature. The most influential and potent zeitgeber of the mammalian circadian system is light and secondary to that is feeding schedule. Consequently, in addition to genetic perturbations, many animal studies to date focus on altering 12 hour light/dark cycles or feeding schedules in order to investigate the effects of disruption of the circadian system (1).

The suprachiasmatic nucleus (SCN), referred to as the central or master clock, is a cluster of about 20000 neurons that is located in the hypothalamus of the brain. The SCN transduces light-driven signals from melanopsin, a G-protein-coupled receptor (GPCR) in the retina, into electrical and hormonal signals to other regions of the brain and body in order to entrain circadian rhythms of many different biological processes, such as the sleep-wake cycle (7). Clocks located outside of the SCN are known as peripheral clocks. As noted above, clocks are present in every cell and are often coordinated at the organ level. Peripheral clocks that can act independently of the SCN may be present in every tissue and have been identified in most organs, including the metabolic, cardiovascular, reproductive, endocrine, immune, and gastrointestinal systems. Input from non-photic zeitgebers act upon these peripheral clocks which, consequently, feed back to the SCN to align the master clock with peripheral clocks (8). The interplay between the central clock and peripherical clocks therefore have implications on not only biological processes but also the pathophysiology of various diseases.

## The Transcription Translation Feedback Loop

At the cellular level a series of TTFLs make up the molecular circadian machinery that control the expression of clock-controlled genes (CCGs) and lead to rhythmic cycling of gene expression and, consequently, behavioral and physiological processes (Figure 1). The core clock transcriptional activators, Brain and Muscle ARNT-Like 1 (BMAL1) and Circadian Locomotor Output Cycles Kaput (CLOCK), form a heterodimer and act upon conserved enhancer "E-box" elements of the promoters of CCGs to initiate their transcription. The set of genes with E-box elements include the negative regulators of the core clock, Period 1/2 (PER1/2) and Cryptochrome 1/2 (CRY1/2) (8). Interestingly, CLOCK itself does not exhibit rhythmic expression but its circadian control and activity are dependent upon BMAL1 in that its nuclear accumulation is BMAL1-dependent (9). Very similarly, PER1/2 are dependent upon CRY1/2 for their nuclear accumulation (10). Following their translation and accumulation in the nucleus, the PER and CRY heterodimer suppresses the transcriptional activity of BMAL1::CLOCK, likely by recruiting Casein Kinase 1 d (CKd), resulting in the phosphorylation of the transcriptional complex and dissociation from the E-box (11). This serves as a negative feedback loop in that PER::CRY suppress their own transcription. The decrease in *PER1/2* and *CRY1/2* transcripts combined with the degradation of PER1/2 and CRY1/2 proteins relieve the suppression of BMAL1::CLOCK transcriptional activity, thereby reinstating CCG output and generating a

new cycle of oscillation (8). Besides this core negative feedback loop, there are additional loops that are generated by positive and negative regulators of *BMAL1* transcription - Retinoic Acid-Related Orphan Receptor α/β/γ (RORα/β/γ) and REV-ERBα/β. The positive regulator RORα/β/γ is encoded by *RORA, RORB*, and *RORC*, respectively. The negative regulator REV-ERBα/β are encoded by the Nuclear Receptor Subfamily 1 Group D Member 1/2 (*NR1D1/2*) genes. RORs and REV-ERBs compete for interaction with the ROR response element (RORE) motif on the *BMAL1* promoter. RORs promote *BMAL1* transcription while REV-ERBs interact with Nuclear Repressor Corepressor (NCoR) to suppress *BMAL1* transcription. As with *PER1/2* and *CRY1/2*, BMAL1::CLOCK control the expression of RORs and REV-ERBs by acting upon the E-boxes of their gene promoters (12). In addition to their positive and negative regulators, BMAL1::CLOCK's transcriptional targets include many proteins including other transcription factors such as Differentiated Embryonic Chondrocyte Gene 1/2 (DEC1/2), D-box Binding Protein (DBP), Hepatic Leukemia Factor (HLF), Thyrotroph Embryonic Factor (TEF), and E4-Binding Protein 4 (E4BP4) (8).

Post-translational modifications and protein turnover add an additional layer of regulation to clock components in order to generate a period length of roughly 24 hours in gene expression cycling. Following synthesis in the cytoplasm, BMAL1, CLOCK, CRY1/2, and PER1/2 nuclear translocation and heterodimerization are regulated by their phosphorylation status (12). For example, phosphorylation of CLOCK at Serine-38 and −42 is important for its transcriptional activity, while phosphorylation at Serine-845 by AKT inhibits its nuclear localization, and phosphorylation at Serine-427 by Glycogen Synthase Kinase-3 Beta (GSK-3β) results in its degradation (13, 14). Adenosine Monophosphate-Activated Protein Kinase (AMPK) phosphorylates and destabilizes CRY1/2. This event is then followed by the subsequent ubiquitination of CRY1/2 by the Skp1/Cullin/F-box protein (SCF) E3 ubiquitin ligase F-box/LRR-repeat protein 3 (FBXL3) complex ($SCF^{Fbxl3}$), resulting in the proteasomal degradation of CRY1/2 (8). However, it is important to note that, like CLOCK, phosphorylation at some residues of CRY1/2 results in degradation while phosphorylation at other residues affects its subcellular localization (15). PER1/2 is phosphorylated by CK1δ/ε, which leads to the recruitment of a complex containing SCF and b-Transducin Repeat-Containing Protein, another F-box protein, ($SCF^{b\text{-}TrCP}$) and, consequently, to the ubiquitination and degradation of PER1/2 (8). CK2 is another circadian related kinase that phosphorylates both BMAL1 (on Serine-90), which results in its nuclear accumulation, and PER2, which leads to either its accumulation in the nucleus or its degradation after additional phosphorylation by CK1d/e (16, 17). Such events suggest that there is a defined ratio in the level of phosphorylation and/or the site of phosphorylation of clock proteins that determines their subcellular localization, stabilization, or degradation.

The dynamics of transcriptional activation and/or repression, protein subcellular localization, and protein degradation as a whole generates oscillations in circadian CCG expression and, consequently, rhythms in behavioral and physiological processes.

## CLOCK AND CANCER

A growing body of evidence reveals that the circadian clock is tightly linked to cancer on multiple levels. Early evidence came from epidemiological studies showing that the

disruption of circadian rhythms by factors such as shift work was associated with increased risk of breast cancer (18, 19), later supported by additional epidemiological data confirming the association between shift work and cancer risk (20-22). This evidence motivated the International Agency for Research on Cancer (IARC) to list “shift work leading to a disruption in circadian rhythm” as a probable human carcinogen (23). Although this connection remains controversial, this connection inspired researchers to investigate the direct correlation between the clock and cancer using prospective physiological and genetic experiments in animal models. For example, chronic jet lag mimicked by shifted lighting or SCN ablation in mice can induce spontaneous hepatocellular carcinoma (HCC) (24, 25). In mice that harbor mutated *p53* or *Ras*, alteration or knock-out of core clock genes such as *Bmal1* and *Per* can accelerate the initiation and progression of multiple cancers (26, 27). Epidemiological and animal model studies are reviewed in detail by Pariollaud et al. (28). More recently, a pan-cancer analysis of The Cancer Genome Atlas (TCGA) showed that alteration of clock genes at transcriptional and genetic levels are pervasive in cancer, and transcriptional dysregulation is strongly associated with survival. Furthermore, the transcription of clock genes and many drug target genes are closely associated with each other (29). This work underscored the clinical significance of clock genes in cancer treatment.

Although a broad association between a disrupted clock and tumorigenesis is well established, the detailed molecular relationship remains elusive. First, it is clear that many cancer cells harbor a disrupted clock machinery where the circadian rhythm of the cells is dampened or completely diminished, but no data thus far have argued clearly whether the disruption of the clock is the cause or effect of malignant cell transformation. Furthermore, in certain types of cancer such as glioblastoma (GBM) the circadian rhythm in tumor cells remains intact and is in fact required for the maintenance of the disease. Second, the isoform-specificity, activity and function of the clock components highly depend on their tissue background, which implies that different roles may be played by the clock in cancers originating from different organs. This explains how certain clock genes can act as tumor suppressors in some tumor types, but oncogenes in others. Third, because of the essential role of the circadian clock in regulating cellular physiology, the downstream processes regulated by the clock machinery in the cells are also broad, weaving complex regulating networks and making it difficult to build clear and definite relationships. The interplay between clock components and the generally-recognized hallmarks of cancer (summarized in Figure 2) have been recently reviewed by Sulli et al. (30). More detailed studies are being carried out to scrutinize the roles of clock molecules in cancer. Here we reviewed some current studies that elucidate the roles of core molecules of the clock machinery in cancer.

## The Core Clock in Cancer

BMAL1 and CLOCK are the central modulators of circadian output in cells and their pathological functions in cancer are the most thoroughly studied so far. BMAL1::CLOCK was initially suggested to be tumor suppressive. Low expression of *BMAL1* is reported to be associated with tumor progression and poor diagnosis in melanoma, pancreatic adenocarcinoma, and breast cancer (31-33). Consistently, overexpression of *BMAL1* in HCC, osteosarcoma, ovarian and hematologic cancer cells reduced tumor cell growth, and

BMAL1 knockout in hamsters results in HCC (24, 34-37). In addition, it is becoming clear that BMAL1::CLOCK works differently across organs by interacting with tissue-specific factors. The molecular mechanism of this tissue-specific control has been exemplified by the liver, where the Hepatic Nuclear Receptor 4a (HNF4A) was shown to repress the BMAL1::CLOCK transcriptional activity, adding a layer of modulation to the circadian network (38). HNF4A and BMAL1 seem to collaboratively regulate the proliferation of HCC cell lines, pointing out the functional role of tissue-specific circadian clocks in carcinogenesis (34). In acute myeloid leukemia (AML) and GBM, BMAL1 and CLOCK are essential for the survival of cancer stem cell (CSC) population. In AML, knock down of *BMAL1* and *CLOCK* led to stem cell differentiation and disease regression in a murine model, while keeping physical hematopoiesis intact (39). This selective role of BMAL1::CLOCK was also observed in GBM, where more details were revealed (40). Glioblastoma stem cells (GSCs), normal neural stem cells (NSCs), and non-malignant (NM) neuronal cultures excised from epilepsy patients all display rhythmic expression of *BMAL1* but disruption of *BMAL1* by shRNA only specifically blocked cell proliferation of GSCs, showing that GSCs have a specific and exquisite reliance on BMAL1 activity for proliferation. GSC, but not NSC, self-renewal was also impaired upon *BMAL1* or *CLOCK* disruption. This observation was explained by chromatin immunoprecipitation sequencing (ChIP-seq) analysis, which showed that in GSCs, BMAL1 had largely skewed binding activities compared to NSCs, including newly gained binding sites and motifs that are enriched in genes involved with glucose and lipid metabolism. Functional assays confirmed that BMAL1 and CLOCK indeed maintained metabolic homeostasis in GSCs. These data highlight epigenetic regulation and metabolism as primary processes regulated by the clock proteins in cancer. Disruption of *BMAL1* or *CLOCK* or BMAL1::CLOCK transcriptional activity by shRNA or small molecules drastically reduced the tumor burden and improved survival in mice model of GBM, indicating that BMAL1::CLOCK-fueled tumor progression is a viable target for anti-cancer therapy (40). In an independent study gain-of-function screen of epigenetic regulators, CLOCK was shown to enhance GSC self-renewal. CLOCK::BMAL1 induced the recruitment of immune-suppressive microglia to the GBM microenvironment and triggered pro-tumor immune activity, hence helping tumor maintenance and progression. Depletion of *Clock* improved the survival in a GBM murine model (41). These studies underscore the role of the clock machinery in cancer and suggest there is great potential for clinical benefits as a result of directly targeting the clock to treat cancer.

## Repressors of the Core Clock in Cancer

The negative regulator arms of the clock are also clearly implicated in cancer etiology. Levels of the PER family genes were found repressed in clinical samples from many cancer types, including *PER1* in glioma, stomach, non-small-cell lung cancer (NSCLC), breast and prostate cancers, *PER2* in lymphoma, leukemia, lung, stomach, and breast cancers, as well as *PER3* in colorectal cancer (CRC) (42). Interestingly, increase in *PER3* expression was observed in patients with AML and acute lymphoid leukemia (ALL) in remission, but not in those who relapsed after treatment, suggesting that upregulation of *PER3* is associated with better clinical outcome and may have a therapeutic potential in acute leukemia (43). Reduced *PER1* and *PER2* expression was associated with shorter survival in glioma and

gastric cancer. Accordingly, overexpression of *PER1* in cell lines can suppress tumor growth and induce apoptosis. Based on reporter assay in cultured cell lines, PER1 was shown to bind to androgen receptor (AR) and inhibited AR-mediated gene activation thus repressing prostate cancer cell growth (44). PER2 suppresses estrogen receptor-a (ERa) transcriptional activation and is required for proteasomal-mediated degradation of ERa in the MCF-7 breast cancer cell line. Furthermore, addition of 17-b estradiol (E2) induces *PER2* expression and *PER2* overexpression results in inhibition of growth and colony formation and induced apoptosis, implying the clock's potential role as part of a feedback mechanism in ER+ breast cancer (45). In addition, PER2 may act as a negative regulator of epithelial-mesenchymal transition (EMT) in normal mammalian epithelial cells by interacting with OCT1 (46).

The nuclear receptor REV-ERBs are relatively less studied in cancer. High expression of REV-ERBβ was reported to be associated with poor overall survival in HCC (47). In a cell line study, REV-ERBα was shown to inhibit the proliferation of gastric cancer cells by regulating glucose metabolism and the pentose phosphate pathway (48). Remarkably, pharmacological activation of REV-ERBs was reported to induce apoptosis by regulating autophagy and *de novo* lipogenesis and to be lethal to multiple types of tumor cells, including brain cancer, CRC, breast cancer, leukemia and melanoma (49).

The CRY family genes also play an apparently complex roles in many cancer types, perhaps explained by the differential role and tissue expression of the two isoforms, CRY1, and CRY2. Single nucleotide polymorphisms (SNPs) in *CRY1* were associated with higher risk of breast cancer. Increased *CRY1* in CRC cells correlated with tumor advancement and poor prognosis (50). Recently, it was found that *CRY1* expression is regulated by androgen hormones in that androgen stimulation via the addition of dihydrotestosterone (DHT) leads to AR binding to the *CRY1* locus in prostate cancer cells. CRY1 was found to regulate DNA repair, homologous recombination, and $G_2$/M transition in the cell cycle, thereby promoting prostate cancer cell survival and metastasis (51). By contrast, a high expression levels of *CRY2* correlated with better survival in breast cancer, and, consistently, a low level of *CRY2* was associated with shorter survival in HCC (29, 52). Expression of *CRY2* was also found to be lowered in papillary and follicular thyroid carcinoma (53). These data imply a tumor suppressive role of CRY2. On the other hand, ablation of both *Cry1/2* seems to suppress tumor development and improve prognosis in mice with mutated *p53*, implying that CRY2 can also be tumorigenic (54). Knocking out both *Cry* genes in hamsters resulted in development of HCC (24). The role of CRY2 in tumor suppression may involve its direct regulation of the oncogene MYC which is discussed in the following section.

The expression of core clock genes and negative regulators of the clock has been also found to be dysregulated in esophageal and cervical cancer cell lines compared to their normal counterparts, with low levels of *CLOCK*, *CRY1*, and *RORA* expression in cancer cells due in part to promoter hypermethylation. Notably, overexpression of *CLOCK* and *PER2* attenuated cell proliferation while activation of RORα and REV-ERBα via small molecule agonists (SR9011 and SR1078, respectively) led to increase in apoptosis in cervical and esophageal cancer cells, with a lesser effect on non-cancerous control cells (55).

### MYC and the Clock

MYC is one of the most well-known oncogenes and its abnormal activation is pervasive in cancer, being deregulated (normally overexpressed) in many cancers. Like BMAL1 and CLOCK, MYC has basic helix-loop-helix (bHLH) structure with a high affinity to E-box motifs. Therefore, it is not surprising that functions of MYC and clock genes are correlated. Cellular studies showed that MYC can disrupt circadian oscillations in at least two ways, by either activating REV-ERBs to suppress *BMAL1* and *CLOCK* transcription or repressing *BMAL1* and *CLOCK* directly in complex with Myc-Interacting Zinc Finger Protein 1 (MIZ1) (56, 57). These results provide a potential mechanism for how the clock is disrupted during tumorigenesis. Conversely, MYC is also regulated by the clock network. MYC expression oscillates in a 24-hour period and the dynamics of the MYC protein is directly regulated by clock proteins. CRY2 can promote the ubiquitylation of c-MYC by acting as a co-factor of FBXL3, leading to MYC degradation. Loss of *Cry2* stabilizes c-MYC and enhances cell transformation and proliferation in mouse fibroblasts (58). CRY modulation is therefore potentially protective against malignant transformation and is thus a potential molecular target for cancer therapy.

The increasing molecular connections between cancer and the clock machinery give exciting inspiration for designing strategies to target clock components for the treatment of cancer.

## TARGETING THE CIRCADIAN CLOCK IN CANCER

BMAL1 and CLOCK are transcription factors that are incredibly challenging to target in the human body due to general difficulties in targeting protein-DNA or protein-protein interactions that arise with transcription factors. Consequently, various small pharmacological molecules that either inhibit or promote the activity of the negative regulators of the clock have been developed in order to enhance or suppress BMAL1::CLOCK transcriptional activity. To date, numerous clock compounds have been applied as potential therapeutic agents in a variety of diseases, including cancer (Table 1). As noted above, BMAL1 and CLOCK can either play an oncogenic or tumor suppressive role depending on the cancer type and the downstream targets of BMAL1::CLOCK transcriptional activity that are actively driving tumorigenesis. It is therefore imperative to account for the specific functions of these compounds before utilizing them to target cancer cells.

### REV-ERB Agonists

REV-ERB agonists have perhaps the richest history amongst the existing collection of clock-targeting small molecules. GSK4112, or SR6452, was the first synthetic REV-ERB agonist to be identified and targets both REVERB isoforms, mimicking the action of heme, the physiological ligand for REV-ERBs. It was able to reset circadian rhythms and regulate metabolic pathways but displayed low systemic exposure *in vivo* (59). GSK4112 derivatives SR9009 and SR9011 display improved potency, efficacy, and pharmacokinetic properties compared to GSK4112 and have been thoroughly studied in numerous disease models (60). They have been found to be potent against a number of different cancer types containing a variety of different oncogenic drivers (40, 41, 49, 61). Notably, SR9009 and SR9011 have

both been reported to not display overt toxicity in animal models (60). It is important to note, however, that high concentrations were needed to observe anti-tumor effects in *in vivo* experiments, suggesting that more potent derivatives of these agonists may need to be developed for therapeutic potential in the clinic (49). It has been reported that SR9009 may display REV-ERB independent effects given that SR9009 still retained its activity in mouse hepatocytes and embryonic stem cells under *Nr1d1/2* (REV-ERBα/β) knockout conditions (62). In previous studies, however, loss of REV-ERBs led to attenuation of SR9009 treatment effects in various disease models (49, 60). Given such findings, SR9009 may not be completely ruled out for pharmacological modulation of REV-ERBα/β activity, but investigators must properly determine whether SR9009 exclusively displays effects on REV-ERBα/β in the disease, tissue, and model of interest in their studies.

Given that SR9009 has an improved but still sub-optimal pharmacokinetic profile and has potential off-target effects, emerging novel REV-ERB agonist scaffolds may pose to be better options for future cancer studies and clinical applications and further *in vivo* investigation of these scaffolds are pertinent moving forward especially in cancer types and other diseases where BMAL1::CLOCK activity are drivers of disease pathophysiology.

## REV-ERB Antagonists

SR8278 was the first identified REV-ERB antagonist with a structure similar to GSK4112 but it competes for heme rather than substitutes for it and thus represses REV-ERBα/β target transcription rather than activates it. SR8278 enhances luciferase reporter activity of target genes that are normally repressed by REV-ERBα/β: *BMAL1*, Glucose 6-Phosphatase (*G6Pase*), and Phosphoenolpyruvate Carboxykinase (*PEPCK*). SR8278 inhibited the repressive transcriptional activity of REV-ERBα/β at a dose dependent manner. Interestingly, SR8278 treatment of HCC HepG2 cells led to a significant increase in the mRNA levels of *G6Pase* and *PEPCK*, genes that play tumor suppressive roles in HCC and renal cell (RCC) carcinomas (63).

ARN5187 is a REV-ERB antagonist that exclusively interacts directly with the ligand-binding domain (LBD) of REV-ERBβ. Treatment of BT-474 breast cancer cells lead to increase in *BMAL1*, *PER1*, and *PEPCK* expression levels in a dose dependent manner. ARN5187 also inhibited autophagy by blocking lysosomal function and preventing autophagolysosome maturation and increased cleaved Poly[ADP-ribose] Polymerase (PARP) levels. REV-ERBβ levels were higher than REV-ERBα in breast, liver, prostate, melanoma, and colon cancers, suggesting that specific inhibition of REV-ERBβ may be a promising therapeutic strategy for these cancer types (64).

As REV-ERBs were found to play a role in inflammatory responses, additional studies will need to be conducted on the action and pharmacokinetic properties of REV-ERB antagonists in inflammatory disease as well as cytotoxicity and chronic inflammation in cancer (65).

## ROR Agonists

SR1078 was identified to be a direct agonist of RORα/γ in a biochemical screen. Co-transfection assays in which Human Embryonic Kidney (HEK) 293 cells were transfected with RORa or g and a luciferase reporter driven by the mouse promoter of *G6Pase*, a

target of RORs, showed that addition of SR1078 was able to significantly increase reporter activity. This increase was lost when the RORE domain was mutated in the *G6Pase* promoter, indicating compound specificity for RORα/γ. The agonistic effects of SR1078 were confirmed by treating human liver cancer HepG2 cells with the compound, which resulted in 2 and 3-fold increase in *G6Pase* and Fibroblast Growth Factor-21 (*FGF21*) mRNA levels, respectively. Intraperitoneal injection of SR1078 into mice showed that the compound displays acceptable pharmacokinetic properties and increased expression of ROR target genes in the liver (66). Interestingly, HepG2 cells treated with SR1078 showed an increase in p53 protein levels, expression of p53 target genes, and apoptosis as indicated by the increase in fraction of cells in sub-$G_1$ phase (67).

Nobiletin (NOB) is another direct agonist of RORα/γ identified through radioligand binding assays in which NOB displayed robust competitive binding to RORα/γ LBD. NOB increased luciferase reporter activity driven by the mouse *Bmal1* promoter at a dose dependent manner in HCC Hepa1-6 cells. This effect of NOB was lost when either the RORE element of the *Bmal1* promoter was mutated or *RORA/C* were knocked down. Pharmacokinetic studies of NOB revealed significant plasma, liver, and brain exposure and NOB treatment significantly increased expression of several ROR target genes in mice (68). Across a variety of cancer cell types, NOB has been found to induce apoptosis and cell cycle arrest, suppress EMT, inhibit many oncogenic drivers, upregulate tumor suppressors, and increase chemotherapy sensitivity (69).

The specificity and favorable pharmacokinetic properties of these ROR agonists suggest that they can be applied in future *in vivo* studies for disease treatment in which BMAL1 transcription and transcriptional activity might be advantageous.

## CRY Stabilizers

KL001 was shown to cause period lengthening and amplitude reduction of luciferase activity in *mBmal1-dLuc* reporter cells when it was identified in a cell-based phenotypic screen of 60000 structurally unique compounds (70). KL001 was able to lengthen the period and amplitude of signal in NIH-3T3 fibroblast cells transfected with *mBmal1-dLuc* or *mPer2-dLuc* reporters as well as mice SCN and lung explants containing a $mPer2^{Luc}$ knock-in reporter. KL001 was found to directly interact with CRY1/2 and stabilized their protein levels by inhibiting FBXL3 mediated ubiquitination of CRY1/2 by occupying the flavin adenine dinucleotide (FAD) binding pocket. KL001 treatment impacted hepatocyte glucose synthesis and is therefore promising for treatment of metabolic disorders (70). There has been an undertaking to identify derivatives of KL001 to improve upon its potency for different disease applications and to improve on its pharmacokinetic characteristics (71, 72). Application of KL001 was well as SHP-656, a derivative of KL001 with increased bioavailability, was found to specifically target GSCs *in vitro* compared to normal neuronal cells by downregulating stemness genes and inducing apoptosis. SHP-656 treatment of mice inoculated with GSCs was able to extend their survival (40).

Recent work was able to identify CRY stabilizers that are specific for either one of the two CRY isoforms, and these pharmacological molecules are structurally different from any other identified clock compound identified thus far (73, 74). The selectivity for CRY

stabilizers for different CRY isoforms will be a powerful tool to investigate the similarities and differences between the CRY isoforms, their functional roles, and how the regulation of the CRYs play a role in a variety of diseases, including cancer.

## CRY Inhibitors

KS15 was identified from a two-step cell-based screening method of 1000 different compounds that utilized BMAL1::CLOCK heterodimer driven luciferase reporter or an artificial E-box luciferase reporter in cultured cell lines. Addition of KS15 obstructed CRY1/2-mediated binding and transcription repression allowing for the activation BMAL1::CLOCK E-box-mediated transcription and resulted in the increase of *Per1/2* and *Nr1d1* (REV-ERBα) mRNA levels, all of which is lost under *Cry1/2* knockout conditions (75). Treatment of MCF-7 breast cancer cells with KS15 induced changes in the expression of cycle-cell regulators and pro-apoptotic genes resulting in cell cycle arrest and induction of apoptosis and repressed proliferation in *in vitro* wound healing assays. KS15 led to an increased sensitivity to doxorubicin and tamoxifen treatment, indicating that KS15 increases the chemosensitivity of some breast cancer cells (76). Optimization of KS15 for *in vivo* application may result in a new therapeutic strategy for treatment of different cancers that is linked to CRY activity.

## CK1 Inhibitors

Longdaysin was identified from a screen of about 120000 compounds using U2OS osteosarcoma cells containing a *mBmal1-dLuc* reporter and causes a strong period-lengthening effect (77). Longdaysin displayed consistent dose dependent response in mice fibroblasts and lung and SCN explants containing a $mPer2^{Luc}$ knock-in reporter and in zebrafish harboring a *per3-luc* reporter. It was revealed that CK1α/δ and ERK2 are both targets of longdaysin. Treatment of U2OS *mBmal1-dLuc* reporter showed that longdaysin strongly upregulated the amount of PER1 protein by influencing interaction of CK1α/δ with PER1 (77). Longdaysin attenuated Wnt/β-catenin signaling by blocking LDL Receptor Related Protein 6 (LRP6) and Dishevelled-2 (DVL2), reduced levels of active and total β-catenin, and decreased the expression of Wnt target genes in Hs578T and MDA-MB-231 breast cancer cells. *In vitro* and *in vivo* breast cancer studies demonstrated that longdaysin suppressed colony and sphere formation, cell migration and invasion, expression of stemness markers, and tumor growth and proliferation (78). A derivative of longdaysin, NCC007, has been shown to have stronger period effects, improved $IC_{50}$'s against CK1α/δ, and lengthened periods of *in vivo* mouse behavioral rhythms, suggesting that further studies of this compound can prove to be more potent against cancer cells (79).

IC261 was first identified through a small molecule screen for antagonistic activity against CK1α/δ/ε in the presence of low and high concentrations of ATP, the nucleotide substrate of CK1. IC261 selectivity inhibits all CK1 isoforms in comparison to protein kinases that are unrelated to CK1 (80). Studies using MEF and C57MG mammary epithelial cells demonstrated that IC261 treatment leads to cell cycle arrest at the post-mitotic $G_1$ phase in a p53-dependent manner while cells that lacked p53 overrode checkpoint control and had high concentrations of DNA, resulting in the development of micronuclei. IC261 caused

centrosome amplification, resulting in multipolar mitosis, and induced apoptosis regardless of p53 status (81).

SR-2890 and SR-3029 are two CK1δ/ε specific inhibitors that were found to have potent anti-proliferative activity in an MTT assay against the A375 melanoma cell line. They also displayed suitable *in vitro* and *in vivo* pharmacokinetic properties and activity in experiments that assessed microsome stability, cytochrome P450 inhibition, and $IC_{50}$ against CK1δ/ε. SR-3029 also exhibited brain penetration, which suggests its potential for treatment of brain cancers (82). In separate studies, SR-3029 showed anti-tumor effects across breast cancer subtypes (83).

Additional studies will need to be performed to elucidate the value of CK1 inhibitors as chronotherapeutic agents for disease modulation but pharmacological targeting of CK1 has proven to be a promising anti-cancer therapy option to pursue in a number of different cancer types.

## CK2 Inhibitors

CK2 is another clock related protein kinase that has important functions in cell growth and apoptosis and, consequently, tumorigenesis. GO289 was identified to be a period lengthening compound through a chemical screen using U2OS *mBmal1-dLuc* reporter cells. Target identification and *in vitro* kinase assays revealed that GO289 potently inhibited CK2 and only had minimal effects on other kinases (84). GO289 was found to inhibit phosphorylation of PER2. GO289 was identified to have anti-cancer effects on human renal-cell carcinoma (RCC) lines, Caki-2, A498, and 769-P, and mice harboring MLL-AF9 based AML tumors. Researchers also found that GO289 had less potent effects on other RCC cell lines and, unlike AML stem cells which have intact circadian rhythms, AML cancer cells themselves have a disrupted circadian clock. These findings suggest that CK2 regulates growth depending on cell type and application of GO289 must be thoroughly investigated in the cancer model of interest (39, 84). There have been other identified and well-studied CK2 inhibitors including TBB, DMAT, and CX-4945 (Silmitasertib), which have displayed clinical effects on the circadian clock and are currently being investigated in a number of cancer clinical trials, however, it has to be noted that they do not display optimal selectivity against CK2 (84, 85).

## GSK-3β Inhibitors

As noted above, phosphorylation of CLOCK Serine-427 by GSK-3β results in its degradation. There is emerging evidence highlighting aberrant activation of GSK-3β in certain cancer types, resulting in sustained survival, stemness, proliferation, invasion, and insensitivity or resistance to chemoradiation. More specifically, GSK-3β was found to have increased expression and deregulated activity due to changes in phosphorylation, contributing to tumorigenesis and cancer progression. Genetic or pharmacological inhibition of GSK-3β led to attenuation of survival and proliferation and induced apoptosis in a number of different cancer cells. GSK-3β is thought to primarily function through β-catenin and c-MYC to confer anti-tumor effects (86). Interestingly, compound library screens using the U2OS *mBmal1-dLuc* reporter cell line and LOPAC chemical library identified some

GSK-3β inhibitors to have period shortening effects (87). Further investigation is necessary to elucidate whether these GSK-3β inhibitors mediate anti-tumor effects through attenuation of circadian cycling in addition to targeting key oncogenic pathways.

## EXPERT OPINION AND FUTURE PERSPECTIVES

The growing research on the interplay between the circadian machinery and cancer has been unveiling the promising therapeutic potential of targeting the clock pathway in oncology. However, the biological understanding of the role of clock genes in cancer dynamics has yet to be fully clarified. In fact, based on the available evidence on pre-clinical studies in different cancer types, a unifying theory modelling the circadian biology of cancer cannot be formulated, implying a unique impact of clock pathway alterations according to each specific tumor type. This specificity reflects a differential effect of individual clock genes on the initiation and progression of specific cancers, the complex interaction between the circadian clock and cancer in which cancer-related alterations may in turn disrupt the clock function, and distinct molecular interactions between clock genes and specific oncogenes in each tissue. In this challenging scenario, when approaching the genetic validation of the role of clock genes and the identification of the optimal target for future drug development, a tailored disease-specific approach will be paramount to yield actionable insights. Nevertheless, the strong rationale behind this intriguing therapeutic approach is opening new horizons in cancer research which warrant further exploration. Moving forward, a deeper mechanistic understanding and dedicated studies exploiting *in vivo* cancer models are needed as a proof of concept to lay the basis for the use of clock proteins-targeting compounds as cancer therapeutics.

Notably, targeting the clock machinery may hold great potential to be effective as a combination treatment with available anti-cancer drugs. The link between clock genes disruption and several druggable cancer-related pathways has been established (Figure 2), including angiogenesis, DNA repair, and RAS/MAPK signaling; furthermore, a few studies highlighted the predictive and prognostic impact of single nucleotide polymorphisms in clock genes on treatment outcomes in different cancer types (including CRC and gastric cancer) (88-90). Hence, pharmacological modulation of proteins in the circadian machinery may offer a novel opportunity to improve other targeted treatment efficacy operating through its effects on major cancer-related pathways. To this extent, the connection between the circadian clock and the immune system may offer a valuable opportunity to enhance immunotherapy efficacy and possibly overcome resistance mechanisms. In fact, core components of the molecular clock - most notably BMAL1, CLOCK, and REV-ERBα - also control fundamental aspects of the immune response modulating immune cell development, function, and trafficking, thus impacting cancer immune surveillance (41, 65, 91). Moreover, the core BMAL1::CLOCK transcription apparatus has been shown to play an essential role in the maintenance of stemness in at least two different cancer types (AML and GBM), therefore the use of small molecules targeting the clock to reduce BMAL1::CLOCK activity and inhibit CSCs may be an effective strategy to enhance cellular sensitivity to non-targeted conventional therapy. Further study into these mechanisms and possible synergistic effects of clock-targeting agents may provide fundamental evidence that will allow the design of

rational combination strategies and promote innovative therapeutic advances in the next future.

Several promising compounds targeting core circadian components and clock-related proteins have shown signs of anti-cancer activity and hold potential to be explored as novel anti-cancer agents (Table 1). However, the development of these drugs may face several challenges. A crucial step before moving forward will be the genetic and pharmacological validation of their target specificity and activity in different cancer types. The heterogeneous and sometimes conflicting role of clock genes according to different cancer types will have to be addressed to dissect the molecular mechanism of action and therapeutic efficacy of different compounds in each tumor of interest. An additional issue worthy of attention is that of potential off-target effects which may differentially contribute in varying degrees to the biological effect of these molecules. On a positive note, evidence shows a selective effect of clock targeting agents (particularly REV-ERB agonists and CRY stabilizers) on cancer cells versus normal cells, supporting the tailored activity of these drugs in cancer (40, 49). Another major challenge in the future development of these compounds will be the selection and optimization of the lead molecules for further study, as several new generation compounds are emerging in an effort to improve pharmacological potency and achieve more favorable pharmacokinetic/pharmacodynamic profiles and better bioavailability. The identification of cancer molecular characteristics which could impact clock-targeting agents' efficacy will also be of paramount importance to select the most appropriate models for *in vivo* studies and eventually individualize treatment when moving towards clinical applications. Finally, the safety profile and potential adverse effects of these drugs will have to be carefully assessed in light of the pleiotropic effects of clock genes in human pathophysiology.

## CONCLUSION

The integration of circadian biology into cancer research is leading to the development of a novel therapeutic paradigm with unique potential to leverage the interplay between the circadian machinery and major cancer-related pathways. While extremely promising, further study is needed to fully establish the efficacy of circadian rhythm modulation as a therapeutic approach in cancer and to inform the modeling of circadian rhythm regulation of cancer biology. If successful, targeting of clock components may significantly improve anti-cancer treatment efficacy by exploiting differential strategies and distinct targets across multiple tumor types.

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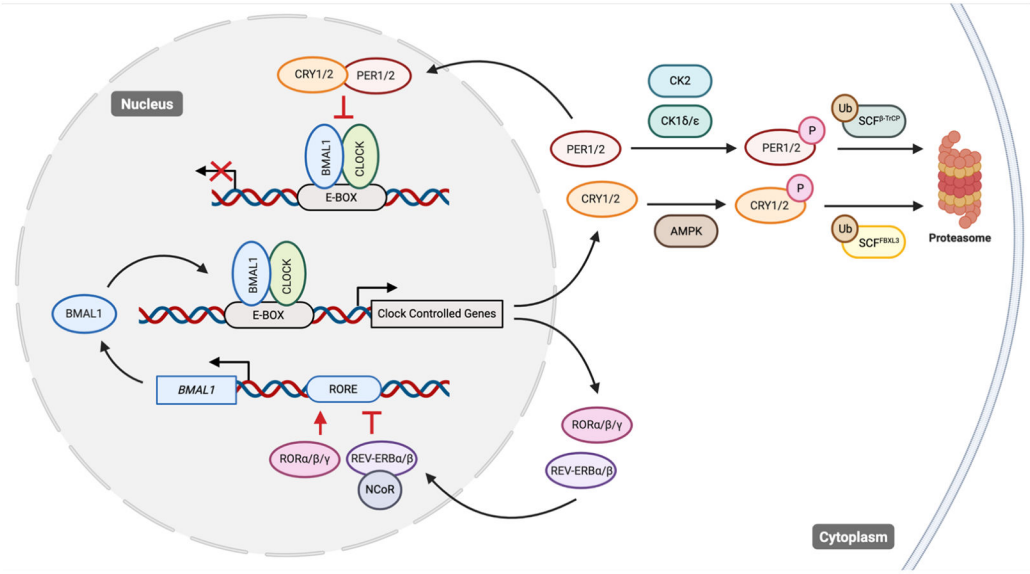


**Figure 1. The Mammalian Circadian Clock.**

The molecular mammalian clock is made up of interlocking TTFLs. The BMAL1::CLOCK heterodimer binds to E-box containing promoters to induce expression of CCGs and their negative regulators, CRY1/2, PER1/2, and REV-ERBα/β, and positive regulators, RORα/β/γ. PER1/2 and CRY1/2 heterodimerize in the cytoplasm and are translocated to the nucleus where they inhibit the transcriptional activity of BMAL1::CLOCK. In doing so, they regulate their expression as well as the expression of other CCGs. Kinases regulate the phosphorylation of these integral clock proteins. PER1/2 is phosphorylated by either CK1δ/ε or CK2 and this is followed by the recruitment of the $SCF^{\beta\text{-}TrCP}$ complex to ubiquitinate and target p-PER1/2 for proteasomal degradation. CRY1/2 is phosphorylated by AMPK and is then ubiquitinated by the $SCF^{FBXL3}$ complex. These events result in CRY1/2 to be targeted for proteasomal degradation. Degradation of PER1/2 and CRY1/2 help to relieve repression upon BMAL1::CLOCK transcriptional activity. RORα/β/γ and REV-ERBα/β on the other hand act upon the RORE promoter element of the *BMAL1* promoter to control transcription of *BMAL1*. RORα/β/γ promote *BMAL1* transcription while REV-ERBα/β interact with NCoR peptides to inhibit *BMAL1* transcription. These TTFLs as a whole generate roughly 24 hours rhythmicity in clock-controlled gene expression. Created with Biorender.com.

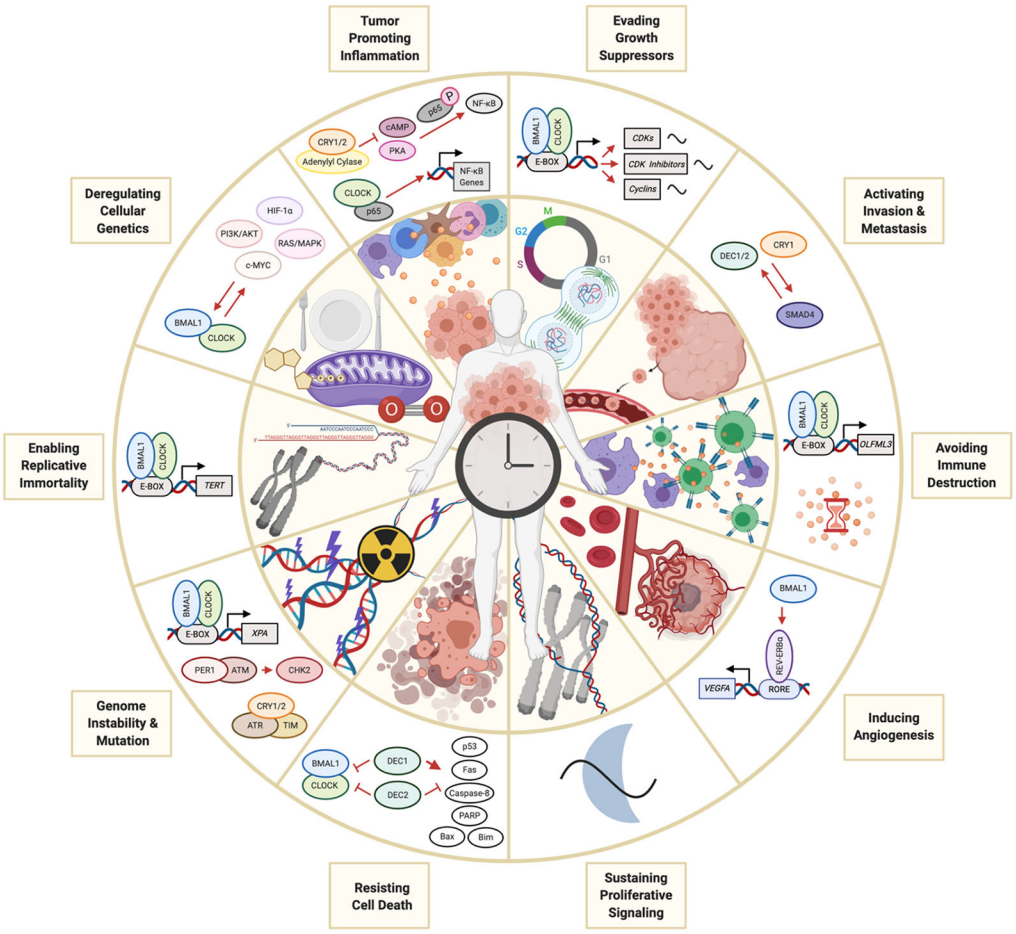


**Figure 2. Circadian Clock Components and Cancer Hallmarks.**

Components of the circadian clock either regulate transcription of tumor suppressors or oncogenes or directly interact with proteins that are part of cell signaling and regulatory pathways that contribute to cancer hallmarks. Cell-cycle regulators such as Cyclin-Dependent Kinases (CDKs), CDK inhibitors, and cyclin proteins exhibit rhythmicity in their expression and some are directly regulated by BMAL1::CLOCK or ROR/REV-ERB transcriptional activity, thereby contributing to proliferation and evasion of growth suppression (30). In pancreatic cancer cells, *DEC1/2* and *CRY1* transcription was dependent on the SMAD Family Member 4 (SMAD4) and activation of the TGFb pathway led to circadian dysregulation and increased cell invasiveness, suggesting crosstalk between clock components and metastasis (92). BMAL1::CLOCK was found to upregulate levels of a chemokine, Olfactomedin-Like 3 (OLFML3), resulting in recruitment of immune suppressive microglia in GBM and separate studies show that disruption of clock components or jet-lag in rodents led to the upregulation of cytokines, resulting in chronic inflammation and immunosuppression (30, 41). An abnormal circadian clock also contributes to T cell exhaustion and immune evasion via global upregulation of inhibitory molecules such as Programmed Death-Ligand 1 (PD-L1) and Cytotoxic T-Lymphocyte-Associated Protein 4 (CTLA-4) (91). BMAL1 regulation of REV-ERBa led to upregulation of Vascular Endothelial Growth Factor A (*VEGFA)* transcription by acting upon its RORE

promoter element and increased proliferation of human CRC cell lines. High *BMAL1* expression in patients was found to be associated with clinical non-response to combined chemotherapy and bevacizumab, an anti-angiogenesis treatment (89). DNA replication occurs at night in many organisms, suggesting tight regulation of these processes by the circadian clock; therefore, modulation of clock components or circadian disruption can lead to sustained proliferative signaling and DNA replication (30). Although both proteins negatively regulate BMAL1 and CLOCK, DEC1 positively regulates while DEC2 negatively regulates proteins involved in apoptosis, suggesting their roles as a circadian-regulated tumor suppressor or oncogene, respectively (93). Not only do components of the DNA damage response pathway and DNA repair mechanisms display rhythms in their mRNA and protein levels but they also directly interact with clock proteins, suggesting daily rhythms in the detection and reparation of damaged DNA. BMAL1::CLOCK may regulate the transcription of Telomerase Reverse Transcriptase (*TERT)* at the E-boxes of its promoter as it displays rhythm in expression, thereby contributing to circadian control of telomerase. Not only does BMAL1 and CLOCK directly regulate key transcription factors, proteins, and signaling pathways that contribute to increased glycolysis in cancer cells and tumorigenesis but they also regulate BMAL1::CLOCK heterodimerization and transcriptional activity. Lastly, BMAL1, CLOCK, and CRY1/2 modulates the function of the NF-kB pathway, one of the key regulators of inflammation, either by directly or indirectly modulating the p65 subunit (30).

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**Table 1.**

Small-molecule Modulators of Clock Proteins for Cancer Therapeutics.

| Compound | Target(s) | Mechanism of Action | Cancer(s) Investigated In | References |
|---|---|---|---|---|
| GSK4112 (SR6452) | REV-ERBα/β | Agonist | Breast, Gastric | (59) |
| SR9009 | REV-ERBα/β | Agonist | Astrocytoma, Breast, CRC, GBM, Leukemia, Melanoma, SCLC | (40, 49, 61) |
| SR9011 | REV-ERBα/β | Agonist | AML, Astrocytoma, Breast, CRC, GBM, Leukemia, Melanoma | (39, 40, 49) |
| SR12418 | REV-ERBα/β | Agonist | - | (65) |
| S 68435-1 | REV-ERBα | Agonist | - | (94) |
| Berberine | REV-ERBα | Agonist | Breast, Cervical, Chondrosarcoma, CRC, Gastric, GBM, HCC, Leukemia, Lung, Intestinal, Melanoma, Multiple Myeloma, NPC, Prostate, SCC | (95) |
| SR8278 | REV-ERBα/β | Antagonist | Gastric, HCC | (63) |
| ARN5187 | REV-ERBβ | Antagonist | Breast | (64) |
| SR1078 | RORα/γ | Agonist | HCC | (66, 67) |
| Nobiletin | RORα/γ | Agonist | AML, Breast, CRC, Fibrosarcoma, Gastric, GBM, HCC, NPC, NSCLC, Neuroblastoma, Osteosarcoma, Ovarian, Prostate, RCC | (69) |
| KL001 | CRY1/2 | Stabilizer | GBM (GSCs), Prostate | (40, 51) |
| SHP-656 | CRY1/2 | Stabilizer | GBM (GSCs) | (40) |
| KL101 | CRY1 | Stabilizer | - | (73) |
| KL201 | CRY1 | Stabilizer | - | (74) |
| TH301 | CRY2 | Stabilizer | - | (73) |
| KS15 | CRY1/2 | Inhibitor | Breast | (76) |
| Longdaysin | CK1α/δ; ERK2 | Inhibitor | Breast | (77, 78) |
| NCC007 | CK1α/δ | Inhibitor | - | (79) |
| IC261 | CK1α/δ/ε | Inhibitor | Breast, CRC, Fibrosarcoma, Gastric, GBM, HCC, Pancreatic, Ovarian, Rhabdomyosarcoma | (80, 81, 96) |
| SR-2890 | CK1δ/ε | Inhibitor | Melanoma | (82) |
| SR-3029 | CK1δ/ε | Inhibitor | Breast, Melanoma | (82, 83) |
| GO289 | CK2 | Inhibitor | RCC, AML | (84) |
| TBB | CK2 (also CDK2; GSK-3β) | Inhibitor | Adenocarcinoma, Breast, Cholangiocarcinoma, CRC, CLL, LCLC, Multiple Myeloma, Prostate, T-ALL | (85) |
| DMAT | CK2 (also PIM1/2/3; PKD1) | Inhibitor | Breast, Prostate | (85) |
| CX-4945 (Silmitasertib) | CK2 (also FLT3; PIM1; CDK1) | Inhibitor | AML, B-ALL, Breast, Burkitt's Lymphoma, Cholangiocarcinoma, CLL, DLBCL, HNSCC, NSCLC, Ovarian, Pancreatic, Prostate, SCC, T-ALL | (85) |
| Chir99021 | GS-K3α/β | Inhibitor | Bladder, Breast, CRC, GBM (GSCs), Melanoma, NSCLC, Ovarian, SCC | (97) |
| Kenpaullone | GSK-3β (also CDK1/Cyclin B; CDK2/Cyclin A; CDK2/ Cyclin E; CDK5/p25) | Inhibitor | Breast, GBM (GSCs), Prostate | (98) |
| 1-Azakenpaullone | GSK-3β | Inhibitor | HCC, GBM, Neuroblastoma | (99-101) |
| Indirubin-3'-monoxime | GSK-3β (also CDK1/Cyclin B; CDK5/p25) | Inhibitor | ALL, Breast, Cervical, CML, CRC, HCC, Lung Adenocarcinoma, Neuroblastoma, OSCC | (102) |