Improved Tumor Control through Circadian Clock Induction by Seliciclib, a Cyclin-Dependent Kinase Inhibitor

Ida Iurisci,1,2 Elisabeth Filipski,1 Jens Reinhardt,3 Stéphane Bach,3 Athos Gianella-Borradori,4 Stefano Iacobelli,3 Laurent Meijer,1 and Francis Lévi1

1Institut National de la Santé et de la Recherche Médicale, U776 “Rythmes Biologiques et Cancers,” Hôpital Paul Brousse, Villejuif Cedex, France; Université Paris Sud, Orsay, France; 2Department of Oncology and Neurosciences, Università ‘G. D’Annunzio,’ Chieti, Italy; 3Centre National de la Recherche Scientifique, Cell Cycle Group, UPS-2682 & UMR-7151, Roscoff Cedex, France; and 4Cyclacel Ltd., Dundee, United Kingdom

Abstract
The circadian timing system and the cell division cycle are frequently deregulated in cancer. The therapeutic relevance of the reciprocal interactions between both biological rhythms was investigated using Seliciclib, a cyclin-dependent kinase (CDK) inhibitor (CDKI). Mice bearing Glasgow osteosarcoma received Seliciclib (300 mg/kg/d orally) or vehicle for 5 days at Zeitgeber time (ZT) 3, 11, or 19. On day 6, tumor mRNA 24-hour expression patterns were determined for clock genes (Per2, Rev-erba, and Bmal1) and clock-controlled cell cycle genes (c-Myc, Wee1, cyclin B1, and CDK1) with quantitative reverse transcription-PCR. Affinity chromatography on immobilized Seliciclib identified CDK1/CDK2 and extracellular signal-regulated kinase (ERK) 1/ERK2, CDK7/CDK9, and casein kinase CK1ε as Seliciclib targets, which respectively regulate cell cycle, transcription, and circadian clock in Glasgow osteosarcoma. Seliciclib reduced tumor growth by 55% following dosing at ZT3 or ZT11 and by 35% at ZT19 compared with controls (P < 0.001). Tolerability was also best at ZT3. Mean transcriptional activity of Rev-erba, Per2, and Bmal1 was arrhythmic in the tumors of untreated mice. Seliciclib induced rhythmic clock gene expression patterns with physiologic phase relations only after ZT3 dosing. c-Myc and Wee1 mRNAs displayed synchronous circadian rhythms in the tumors of control mice receiving vehicle only but not in those of mice given the drug. Seliciclib further enhanced Wee1 expression irrespective of dosing time, an effect that reinforced G2-M gating. Seliciclib also inhibited CK1ε, which determines circadian period length. The coordination of clock gene expression patterns in tumor cells was associated with best antitumor activity of Seliciclib. The circadian clock and its upstream regulators represent relevant targets for CDKIs. (Cancer Res 2006; 66(22): 10720-8)

Introduction
Large epidemiologic studies have shown an increased incidence of breast or colorectal cancers in populations exposed to prolonged shift work, frequent transmeridian flights, or light exposure at night (1–3). These latter conditions profoundly alter circadian rhythms. In addition, a tight relationship has been established between circadian rhythm disruption and quality of life and even survival of cancer patients (4, 5). Taken together, these findings support a close link between the circadian timing system and the cell division cycle, which is frequently deregulated in cancer cells.

The circadian timing system efficiently orchestrates the physiology of living organisms to match environmental or imposed 24-hour cycles (6). Most cells in the brain and peripheral tissues contain a molecular clock consisting of at least 12 specific clock genes in mammals. This molecular clock rhythmically controls the transcriptional activity of ~10% of the genome, ~10% of which are proliferation-related genes (7). Among the 12 genes that constitute the molecular clock, Per2, Bmal1, and Rev-erba play a central role. Thus, mice with a null mutation in these genes displayed profound alterations of the circadian phenotype in constant darkness. Indeed, the rest-activity rhythm was ablated in Per2 or Bmal1 mutants. Rev-erba mutation shortened the period length of this rhythm with wide interindividual variations and dramatically altered the phase-shifting response to light (6, 8, 9). The intracellular clock mechanisms involve interacting positive and negative transcriptional feedback loops that drive recurrent rhythms in the RNA levels of these key components. High levels of Bmal1 mRNA and protein promote the formation of BMAL1: CRYPTOX heterodimers that bind to the E-box sequences in the promoter of clock genes Per2 and Rev-erba and activate their transcription. In turn, Rev-erba negatively regulates Bmal1 transcription, whereas PER2:CRY1 complexes inhibit the transcription of their own genes by interfering with CLOCK:BMAL1 (6, 8, 9).

In previous studies, we found that the circadian expression pattern of core clock genes Per2, Bmal1, and Rev-erba was severely altered in Glasgow osteosarcoma, a rapidly proliferating tumor transplanted in mice (10, 11). Further disruption of the circadian timing system with ablation of the hypothalamic pacemaker or chronic jet lag accelerated malignant progression of this tumor (10–12).

At least three molecular mechanisms link molecular clock with the cycling of cell division. The molecular clock controls Wee1 transcription through an E-box-mediated mechanism (13). Wee1 negatively controls the activity of cyclin-dependent kinase (CDK) 1/cyclin B1, which regulates the G2-M transition. In addition, the BMAL1:CLOCK heterodimers repress c-Myc transcription through E-box-mediated reactions in the c-Myc gene P1 promoter and mPer2 can suppress c-Myc expression indirectly by stimulating Bmal1 transcription (8). In general, knocking down the molecular clock modified transcription patterns of genes involved in the cell cycle regulation, which translated into genomic instability, thus favoring malignant progression or growth (8, 11). Finally, Per1 as well as Per2 and possibly other clock genes also control DNA repair through interactions with ataxia-telangiectasia mutated and mdm2 (14, 15).
Based on the evidence for signaling pathways shared by the circadian clock and the cell cycle (8, 11–16), we have used the CDK inhibitor (CDKI) Seliciclib (also known as R-roscovitine, CYC202; ref. 17) as a pharmacologic tool to investigate the therapeutic relevance of cross-talks between the circadian clock and the cell division cycle in tumors grown in mice. Seliciclib is a 2,6,9-trisubstituted purine in clinical phase II development. This drug competes with ATP for the binding to the catalytic site of CDKs and induces transient growth arrest in both G1/S and G2-M by inhibiting CDK2/cyclin E and CDK1/cyclin B1, respectively (18). It also interferes with transcription by inhibiting CDK7/cyclin H and CDK9/cyclin T, two kinases involved in the phosphorylation of the COOH-terminal repeat domain of RPB1, the largest subunit of RNA polymerase II. It further interacts with the casein kinase 1 (CK1) family (19, 20), which plays a central role in circadian clock function. Indeed, CK1ε or CK1δ mutations or alterations produce circadian disorders of rest-activity or sleep-wakefulness rhythms both in rodents and in humans (6, 21). In particular, phosphorylation S662 of hPER is required for the serial phosphorylation of COOH-terminal serines by CK1ε, and an S662G missense mutation causes familial advanced sleep phase syndrome in a dominant fashion (22).

In the current study, the antitumor efficacy of Seliciclib and its effects on molecular clock and cell division cycle genes in tumor were investigated in mice with Glasgow osteosarcoma, a rapidly growing tumor known to be regulated by the circadian timing system (10–12).

Materials and Methods

The study was conducted in accordance with the guidelines approved for animal experimental procedures by the French Ethical Committee (decree 87-848).

Animals and Synchronization

B6D2F1 male mice, 5 to 6 weeks old, were purchased from Janvier (Le Genest St Isle, France). All mice were synchronized with an alternation of 12 hours of light and 12 hours of darkness in an autonomous chronobiological animal facility (Jouan-Thermo Electron LED S.A.S., Saint-Herblain, France) for 3 weeks before tumor inoculation (10). An adequate synchronization of mice by these lighting regimens was assessed by the occurrence of a normal circadian variation in rectal temperature before treatment initiation. The experiment involved 132 B6D2F1 male mice bearing Glasgow osteosarcoma. Nine days after tumor inoculation, when overall tumor weight reached 100 to 400 mg, Seliciclib (300 mg/kg/d) was given via oral gavage once daily during 5 days to a total of 96 mice at one of three different times referred to light onset [hours after light onset (HALO)], so-called Zeitgeber time (ZT): ZT3, ZT11, or ZT19 (32 mice per dosing ZT). ZT3 and ZT11 were located during the light span when mice usually rest; ZT19 corresponded to the dark span when mice are usually active. Thirty-six mice [12 per circadian time (CT)] received vehicle (50 mmol/L HCl). After the last drug injection, all the animals were exposed to constant darkness during 24 hours, then they were sacrificed at CT5, CT11, CT17, or CT23, and two perpendicularly growing tumors were measured by a caliper and tumors were dissected (Fig. 1). This procedure avoids any masking effect of light on circadian rhythmic patterns and provides a reliable estimate of CT in groups of individual mice within the initial 3 days in constant darkness because the circadian phase of B6D2F1 mice in constant darkness only drifted by 10 to 30 minutes daily (23). The samples (30 mg) were frozen immediately in liquid nitrogen and stored at −80°C until RNA extraction. Circadian expression patterns of mPer2, mBmal1, mRev-erbα, mWee1, mCyclin B1, mCDK1, and mC-Myc were measured with reverse transcription-PCR using LightCycler instrument (Roche Diagnostics, Meylan, France).

Drugs

Seliciclib was obtained from Cyclacel Ltd. (Dundee, United Kingdom). It was diluted in 50 mmol/L HCl and administered via oral gavage once daily during 5 days. Controls received 50 mmol/L HCl.

Tumor Model

Glasgow osteosarcoma tumor was kindly provided by the Research Centre of Aventis Pharma (Vitry sur Seine, France). It was transplanted as bilateral sc. fragments (3 × 3 mm) in each flank of the recipient mouse. Tumor length and width (mm) were measured with a caliper daily and tumor weight (mg) was computed as follows: tumor weight = (length × width²) / 2 (10).

Real-time Quantitative PCR

Total RNA was extracted from frozen tissue specimens of tumor using RNeasy Mini kit (Qiagen S.A., Courtaboeuf, France) according to the manufacturer’s instructions. Contaminating genome DNA was eliminated.
by treatment with RNase-Free DNase Set kit (Qiagen). First-step reverse transcription was done with the High-Capacity cDNA Archive kit (Applied Biosystems, Courtaboeuf, France) according to the manufacturer's instructions. RNA (5 μg) was reverse transcribed using mutiscribe (Applied Biosystems).

Second-step real-time PCR was done in LightCycler using QuantiTect SYBR Green PCR kit (Qiagen). All the PCR primers were obtained from Invitrogen Life Technologies (Cergy Pontoise, France). Primer sequences were designed to avoid the formation of secondary structures with Primer3 normalization buffer. Homogenates were centrifuged for 10 minutes at 14,000 × g at 4°C. The supernatant was recovered, assayed for protein content [Bio-Rad (Marnela Coquette, France) protein assay], and immediately loaded batch wise on the affinity matrix.

Seliciclib and N6-methyl-Seliciclib (control, kinase-inactive analogue) beads were obtained as described (20, 24). Just before use, 10 μL of packed Seliciclib beads were washed with 1 mL of bead buffer and resuspended in 600 μL of this buffer. The tumor extract supernatant (3 mg total proteins) was then added; the tubes were rotated at 4°C for 30 minutes. After a brief spin at 10,000 × g and removal of the supernatant, the beads were washed four times with bead buffer before addition of 60 μL 2× Laemmli sample buffer. Following heat denaturation for 3 minutes, the bound proteins were analyzed by SDS-PAGE and Western blotting or silver staining as described below.

Electrophoresis and Western Blotting

Antibodies. The following antibodies were obtained from commercial sources: anti-CK1α (1:500, 1 hour; BD Transduction Laboratories, Erembodegem, Belgium), anti-CK1ε (1:1,000, 1 hour; Cell Signaling Technology, Beverly, MA), anti-extracellular signal-regulated kinase (ERK) 1/ERK2 (1:500, 1 hour; Santa Cruz Biotechnology, Santa Cruz, CA), anti-CDK9 (1:500, 1 hour; Santa Cruz Biotechnology), and anti-cyclin H (1:3,000, 1 hour; Sigma). Anti-PSTAIRE (1:4,000, 1 hour; Sigma, Saint Quentin Fallavier, France), anti-CDK7 (1:500, 1 hour; Santa Cruz Biotechnology, Beverly, MA), anti-extracellular signal-regulated kinase (ERK) 1/ERK2 (1:3,000, 1 hour; Sigma), anti-CDK7 (1:500, 1 hour; Santa Cruz Biotechnology, Santa Cruz, CA), anti-CDK9 (1:500, 1 hour; Santa Cruz Biotechnology), and anti-cyclin H (1:500, 1 hour; Santa Cruz Biotechnology). Anti-PDXK was generated as previously reported (18), and anti-α/γ/CaMKII (RU16) was prepared and characterized at The Rockefeller University (New York, NY).

Electrophoresis and Western blotting. Following heat denaturation for 3 minutes, the proteins purified with the Seliciclib matrix were resolved by 10% SDS-PAGE (0.7-mm-thick gels) followed by immunoblotting analysis or silver staining using an Amersham Biosciences/GE Healthcare (Orsay, France) SDS-PAGE silver staining kit. For immunoblotting, proteins were transferred to 0.45-μm nitrocellulose filters (20, 24). These were blocked with 5% low fat milk in TBS-Tween 20, incubated for 1 hour with antibodies, and analyzed by enhanced chemiluminescence (Amersham Biosciences).
The PCR product was cloned into pGEX-4T1 (Amersham Biosciences). The fusion protein was expressed in Escherichia coli BL21 (Stratagene, Amsterdam, the Netherlands) after induction with 0.5 mmol/L isopropyl-β-D-thiogalactopyranoside at 32°C for 3 hours. Bacteria resuspended in PBS containing protease inhibitors aprotinin, soybean protease inhibitor, and leupeptin (10 μg/mL each) and 100 μmol/L benzamidine were lysed by sonication or using a French press at 160 bar. After centrifugation at 10,000 g for 10 minutes, the supernatant was recovered and the glutathione S-transferase (GST)-axin fragment fusion protein was purified on glutathione agarose matrix (Sigma) for 1 hour in bead buffer. The beads were washed in bead buffer and stored until further usage on ice.

**Kinase assay buffer.** β-Glycerophosphate (60 mmol/L), 30 mmol/L p-nitrophenylphosphate, 25 mmol/L MOPS (pH 7.0), 5 mmol/L EGTA, 15 mmol/L MgCl₂, 1 mmol/L DTT, and 0.1 mmol/L sodium vanadate.

**CK1 purification and activity determination.** The tumor extract supernatant (0.5 mg/assay) was loaded on glutathione agarose beads saturated with GST-axin and incubated with bead buffer for 1 hour under constant rotation at 4°C. After a brief spin at 10,000 g and removal of the supernatant, the beads were washed thrice with bead buffer and once in kinase assay buffer. For the kinase assays, 50 μmol/L of the CK1-specific peptide substrate RKKAAlGpSAYSTG (pS stands for phosphorylated serine; gift of F. Meggio and L. Pinna, Università di Padova, Dipartimento di Chimica Biologica, Padova, Italy; ref. 24), 15 μmol/L ATP containing 3 μCi/mL [γ-33P]ATP, and kinase assay buffer were added to the beads as well as the indicated concentrations of Seliciclib (with the solvent DMSO added to 10%). The reaction was carried out for 15 minutes at 30°C with frequent mixing, and the reaction was stopped by cooling on ice. The reaction mixture was spotted on phosphocellulose paper (Whatman p81, Oxon, United Kingdom) filters, which were washed four times for 5 minutes in 1% phosphoric acid. Scintillation was measured in a Packard Instruments (Rungis, France) scintillation counter after addition of 1 mL scintillation fluid.

**Statistical Analyses**

For each studied variable, means and standard errors were calculated. Intergroup differences were compared statistically using multiple-way ANOVA. Statistical significance of circadian rhythmicity was validated.
with Cosinor analysis (11). This method characterizes a rhythm by variables of the fitted cosine function best approximating all data. Periods $\tau = 24$ hours and $\tau = 12$ hours were considered a priori. The rhythm characteristics estimated by this linear least squares method include the mesor (rhythm-adjusted mean), the double amplitude (difference between minimum and maximum of fitted cosine function), and the acrophase (time of maximum in fitted cosine function, with light onset as phase reference). A rhythm was detected if the null hypothesis was rejected with $P < 0.05$. Differences in lethal toxicity were compared with $\chi^2$ Fisher's exact test. All standard statistical tests were done using Statistical Package for the Social Sciences version 11.5 for Windows software (SPSS, Chicago, IL).

# Results

CT dependency of therapeutic activity. Treatment with Seliciclib or vehicle was initiated 9 days after tumor inoculation, when the mean tumor weight ($\pm$SE) was 278 mg ($\pm$12; range, 141-458 mg). Daily oral treatment was given to different groups of mice at one of three dosing times separated by 8 hours: ZT3 HALO, ZT11, or ZT19. At the end of treatment, mean tumor weight was reduced by 55% in the animals receiving Seliciclib at ZT3 or ZT11 and by 35% in those treated at ZT19 compared with untreated controls ($P < 0.001$, ANOVA; Fig. 2). Seliciclib toxicity was the lowest following dosing at ZT3 (lethal toxicity rate, 3.1%) compared with ZT11 (6.2%) or ZT19 (21.9%; $P = 0.07$, Fisher's exact test).

Seliciclib induction of clock gene expression rhythms in tumors. No significant rhythmic transcription was found for any of the three clock genes (Rev-erbα, Per2, and Bmal1) tested in the tumors of untreated mice. Seliciclib dosing at ZT3 induced rhythmic expression of Rev-erbα ($P = 0.007$), Per2 ($P = 0.0004$), and Bmal1 ($P = 0.08$) with 5.2-, 3.8-, and 2.4-fold respective peak to trough differences (Fig. 3A-C). The zenith in circadian Bmal1 mRNA accumulation in the tumors of mice treated at ZT3 correlated well with the circadian nadir in Rev-erbα mRNA, a typical feature in healthy tissues (9). In addition, Seliciclib nearly halved the 24-hour mean mRNA expression of Rev-erbα and doubled that of Bmal1 in the tumors of mice treated at ZT3 (Fig. 3D), an effect that was not seen at other dosing times.

The tumor circadian clock was modeled through an adjustment of 24-hour cosine functions to the expression data of the three core clock genes. The tumor clock in untreated mice was clearly disrupted (Fig. 4B) compared with that in healthy liver (Fig. 4A) where the acrophases occur at 5.20 (95% CI, 2.40-8.00) for Rev-erbα, 14.20 (95% CI, 10.20-18.20) for Per2, and 23.40 (95% CI, 21.40-1.40) for Bmal1 (11). In mice given Seliciclib at ZT3, both the double amplitude and the acrophases of the three clock genes in tumor became similar to those in healthy liver; for example, acrophases were respectively located at 5.30 (95% CI, 2.20-8.40), at 11.20 (95% CI, 9.00-13.40), and at 22.40 (95% CI, 19.10-2.10; Fig. 4C). Seliciclib dosing at ZT11 also resulted in double amplitudes in clock gene expression that were similar to those in healthy liver, yet the acrophases were ill placed; for example, at 11.20 (95% CI, 9.50-12.50) for Rev-erbα, at 17.50 (95% CI, 16.40-19.00) for Per2, and at 5.20 (95% CI, 1.10-9.30) for Bmal1 (Fig. 4D). Finally, Seliciclib administration at ZT19 achieved double amplitudes in clock genes that were nearly half of those in mice treated at ZT3 or ZT11. The acrophases were nearly opposite as those found in normal liver; for example, at 18.40 (95% CI, 14.40-23.20) for Rev-erbα, at 23.50 (95% CI, 20.10-3.30) for Per2, and at 12.10 (95% CI, 7.00-17.20) for Bmal1 (Fig. 4E).

Seliciclib alteration of cell cycle gene expression patterns in tumors. c-Myc and Wee1 mRNAs displayed synchronous circadian rhythms in control tumors ($P$ from cosinor = 0.03 and 0.01, respectively) with acrophases occurring at 9.00 (95% CI, 4.30-13.30) and 11.50 (95% CI, 8.20-15.20). Seliciclib treatment at ZT3 advanced the acrophase of c-Myc to 3.50 (95% CI, 23.30-8.10) and ablated Wee1 rhythm (Fig. 5A and B). Both rhythms were suppressed in mice treated at ZT11 or ZT19 (data not shown). No rhythm was

![Figure 4. Cosinor-based model of molecular clock in tumor as a function of Seliciclib dosing time. A, physiologic clock. The adjustment of a cosine curve with a 24-hour period to mRNA expression of Rev-erbα (blue line), Per2 (red line), and Bmal1 (green line) allowed to build a model of the circadian clock in healthy liver of B6D2F1 mice (data from 11). This model establishes physiologic dynamic relations with maxima occurring at CT6 for Rev-erbα, CT15 for Per2, and CT23 for Bmal1. B to E, tumor clock. The same method was applied to clock gene data obtained in the current study for control tumors (B) and for tumors from mice treated with Seliciclib at ZT3 (C), ZT11 (D), or ZT19 (E). Physiologic phase relations between expression patterns of the three clock genes, similar to those in healthy liver, were induced by treatment at ZT3 or at ZT11 but not at ZT19.](cancerres.aacrjournals.org)
observed for CDK1 or cyclin B1 expression in the tumors of control or treated mice (data not shown). Seliciclib doubled the expression of Wee1 (P = 0.002) and CDK1 (P = 0.06) compared with controls and mildly increased cyclin B1, with largest increments in the mice dosed at ZT3 (Fig. 5C).

**Main cell cycle, transcription, and circadian clock target molecules of Seliciclib in tumors.** Affinity chromatography was done on Glasgow osteosarcoma extracts using immobilized Seliciclib, with immobilized CDK-inactive N6-methyl-Seliciclib as a control matrix (Fig. 6A; refs. 20, 24). SDS-PAGE followed by silver staining and Western blotting with various antibodies revealed the expected targets [i.e., CDK1/CDK2 (jointly detected with anti-PSTAIRE), ERK1/ERK2, calmodulin-dependent kinase 2 isoforms, and pyridoxal kinase (PDXK); Fig. 6B]. In addition, this method also identified Seliciclib interactions with CK1ε but not CK1α. Both CDK7, and its activatory cyclin H, and CDK9 were also detected on Seliciclib beads. None of these proteins, except PDXK, interacted with control N6-methyl-Seliciclib agarose (Fig. 6B).

Seliciclib inhibited the catalytic activity of CK1 following affinity purification of Glasgow osteosarcoma fragments from untreated mice. The IC_{50} was 18 μmol/L (Fig. 6C), in agreement with prior studies on other tissue extracts (20, 24).

**Discussion**

CDK pathways represent promising targets as cancer therapeutics (25). Seliciclib, like flavopiridol, another CDKI in clinical testing, has shown activity against several human tumor cell lines both *in vitro* and following engraftment in nude mice. Surprisingly, however, no report mentions any activity of these compounds in the mouse tumor models that are currently used for preclinical testing (26, 27). Overall, clinical trials have revealed modest activity of both compounds against solid tumors (25). Yet a marked efficacy of flavopiridol was recently uncovered in patients with refractory chronic lymphocytic leukemia receiving combined bolus and intermittent infusional delivery (28). These and other results have emphasized the marked schedule dependency of CDKI efficacy, possibly influenced by the cyclic nature of the CDK target pathways themselves. Thus, the rhythmic assembly and dissociation of CDKs and cyclins drive the physiologic course of the cell division cycle, a biological clock in its own. Furthermore, the cell division cycle closely interacts with the circadian clock, which controls several cell and organism functions along the 24-hour time scale (8, 13–16, 29). In particular, the circadian clock controls Wee1 expression, thus gating G2-M transition, a checkpoint that is also largely affected by Seliciclib (13).

Figure 5. Effects of Seliciclib treatment on mRNA expression patterns of clock-controlled cell cycle genes. Relative mRNA levels for c-Myc (A) and Wee1 (B) in the tumors of mice after 5 days of treatment with Seliciclib at ZT3 (solid line) or vehicle (dashed line). The relative mRNA expression of each gene of interest was normalized to that of 36B4. Points, mean of eight to nine (controls) or six to eight (treated) mice; bars, SE. X axis, hatched rectangles, subjective light span; black rectangles, dark span. To better visualize the rhythm during the 24-hour period, data obtained at CT5 were repeated 24 hours later. The circadian rhythms of c-Myc and Wee1 in control tumors (P from cosinor = 0.003 and 0.001, respectively) were abolished in mice treated with Seliciclib. C, twenty-four-hour means of mRNA levels in controls and in mice treated with Seliciclib at ZT3, at ZT11, or at ZT19. Columns, mean of 24 to 30 tumors; bars, SE. Data are expressed as % of mean mRNA levels of controls. In mice treated at ZT3, Wee1 expression was more than doubled compared with controls (P = 0.004, ANOVA; *, P = 0.008, post hoc test). Cyclin B1 was also increased (P = 0.0018, ANOVA). Seliciclib treatment also significantly increased CDK1 mRNA expression at all dosing times (P < 0.0001, ANOVA; *, P < 0.0001, post hoc test).
In our study, we confirmed the antitumor efficacy of Seliciclib in mice with Glasgow osteosarcoma, a rapidly growing mouse tumor. The cellular effects of Seliciclib result from combined actions on several protein targets. Seliciclib arrests cell cycle through the inhibition of CDK1 and CDK2, which respectively gate G2-M and G1-S transitions (17, 26, 30). The cell cycle effects are further reinforced by the inhibition of CDK7/cyclin H, an activator of CDKs, and that of CDK9/cyclin T by Seliciclib. Thus, this drug may also act as a general transcription inhibitor because the two latter molecular targets are essential regulators of RNA polymerase II (31). The transient Seliciclib-induced inhibition of CDK7/cyclin-dependent transcription leads to the down-regulation of short-lived mRNAs and proteins of survival factors Mcl-1, XIAP, and survivin (32, 33). Such broad effects of Seliciclib on the cell cycle and transcription machineries could depend on drug dose and schedule and/or tumor cells characteristics. Our study identified the expected molecular targets of this drug in Glasgow osteosarcoma following purification on Seliciclib-immobilized sepharose beads. These results supported the known mechanisms of antitumor effects of Seliciclib in this tumor model.

A circadian rhythm in Seliciclib tolerability was found with least lethal toxicity corresponding to drug dosing at ZT3, in the early rest span. Highest toxicity occurred when the drug was given at ZT19, near the middle of the activity span. This chronotolerance was consistent with that reported earlier for cytotoxic chemotherapeutic drugs with different mechanisms of action, such as oxaliplatin, irinotecan (34), docetaxel (35), or gemcitabine (36).

Furthermore, the efficacy of Seliciclib against Glasgow osteosarcoma clearly depended on dosing time. After five daily doses, the drug was most active following administration during the rest span compared with dosing during the activity span. Such coincidence of chronoefficacy and chronotolerance was in good agreement with prior results obtained in this and other mouse tumor models (34–37).

The therapeutic relevance of the molecular interactions between the circadian clock and the cell division cycle was investigated. No significant rhythmic transcription of core clock genes *Rev-erb*, *Per2*, and *Bmal1* was found in the tumors of control mice, thus confirming previous observations of damped or suppressed clock gene rhythms in Glasgow osteosarcoma (10, 11). Previous studies also revealed that the more the clock gene patterns were altered, the faster tumors grew and developed (10–12, 14). In the current study, Seliciclib induced rhythmic expression of the three main clock genes in the tumor. This induction persisted for at least 48 hours after the last dose. Treatment in early light, when the drug was most effective, induced near-normal clock gene expression patterns with phase relations similar to those reported in healthy organs. In the liver and in other peripheral organs of mice, the circadian peak in mRNA synthesis usually occurs near the middle of the light span for *Rev-erb*, shortly after light-dark transition for *Per2*, and in the second half of the dark span for *Bmal1* (11). Seliciclib dosing near mid-dark, when the drug was least effective, produced abnormal phase relations between clock gene expression rhythms, a situation that results in a nonfunctional clock.

The ablated rhythms in clock gene expression in the tumor tissue of untreated mice could result from an impairment of the molecular clock within each individual cancer cell or from an altered synchronization of the molecular clocks in each malignant cell. Indeed, individual transformed fibroblasts and hepatoma cells remained self-sustained circadian oscillators with slightly different periods when cultured without any synchronizing signal (38, 39). Even dividing fibroblasts passed information on their own circadian phase to their daughter cells (40). Thus, daily Seliciclib could reset the clocks of malignant cells, an effect also achieved with glucocorticoids and meal timing (11, 39, 41). However, Seliciclib also significantly modified the 24-hour mean expression of *Rev-erb* and *Bmal1* in opposite directions without affecting that of *Per2*, a finding consistent with the down-regulation of *Bmal1* by *Rev-erb* (6, 8, 9). Such effect could not result from the mere synchronization of the molecular clocks in distinct malignant cells. Rather, a direct intervention of Seliciclib on the clock mechanisms itself must be considered. Thus, in mouse liver, this agent both modified the mean 24-hour mean expression of these genes and altered their rhythm pending on dosing time (42).

In the current study, the induction of a functional molecular clock in tumor was associated with the best antitumor activity of the drug. Seliciclib did not alter mean expression of c-Myc but enhanced that of *Wee1*. This effect could decrease tumor cell...
proliferation as a result of improved control of G2-M gating. The enhanced Weel transcription was consistent with the doubling of Bmal expression because Weel is unidirectionally controlled by CLOCK:BMAL1 (13). The observed increase in CDK1 expression likely resulted from a feedback loop where inhibition of CDK enzymatic activity by Seliciclib stimulated CDK mRNA synthesis. Overall, the delivery of Seliciclib at ZT3 both induced near physiologic rhythms in clock gene expression and enhanced the expression of G2-M genes in tumor. Seliciclib dosing at other times induced abnormal rhythms in clock gene expression and/or weaker activation of G2-M gating genes. Two possible mechanisms could account for Seliciclib effects on the circadian clock. Firstly, CK1δ/ε, a key determinant of circadian period (21, 43–45), could be inhibited by Seliciclib (19, 20, 46). Our study further confirmed such effect on CK1δ/ε activity from Glasgow osteosarcoma. Seliciclib more specifically targeted CK1δ, a kinase known to bind to and to phosphorylate Per proteins, leading to their ubiquitination and degradation. Inhibition of CK1δ impairs Per2 degradation and nuclear translocation and results in increased Bmal1 transcription (6, 43), an effect consistent with the average doubling in Bmal expression that resulted from Seliciclib dosing at ZT3 in our study. Secondly, highly coordinated sequential transcription is a major mechanism of circadian rhythms. Seliciclib could affect the generation of circadian rhythmicity through moderate and transient time-dependent inhibition of CDK7 and CDK9. Indeed, the reversible inhibitor of transcription, 5,6-dichloro-β-1-ribobenzimidazole, mimicked the circadian rhythm alteration that was produced with Seliciclib in a mulluscan model (47, 48). Thus, daily Seliciclib could act as a strong resetter of tumor cells that have lost synchrony in functional clocks through transient inhibition of CK1δ within a permissive time window (ZT3 or ZT11). Simultaneous inhibition of CDK7 by Seliciclib would further block the gene expression of CK1δ/ε, which is otherwise constitutively expressed (49).

In conclusion, the antitumor effect of Seliciclib in mice was found to be circadian rhythm dependent, reaching an optimum efficacy when dosing was done near the beginning of the rest span. This maximal antitumor activity coincided with a minimal toxicity. The mechanisms underlying this favorable drug effect involve interactions of Seliciclib with regulators of cell cycle, transcription, and circadian rhythm. Results also suggest that cross-talks between both biological oscillators represent a relevant dynamic target for cancer therapeutics and further support the consideration of appropriate circadian timing for CDKIs.

Acknowledgments

Received 6/7/2006; revised 8/18/2006; accepted 9/20/2006.

Grant support: Association pour la Recherche sur le Temps Biologique et la Chronothérapie (F. Lévy), Association pour la Recherche sur le Cancer (grant N°3342), and Paul Brousse Hospital Villejuif, France (F. Lévy). European Union through the Network of Excellence BioSim, contract no. LSHB-CT-2004-00517 (F. Lévy) and FP6-2002-Life Sciences & Health, PRO-KINASE Research Project (L. Meijer); Canceropole Grand-Ouest, France (L. Meijer); Cyclica Ltd., Dundee, United Kingdom; and Università “G. D’Annunzio,” Chieti, Italy (fellowship to I. Iuriciu).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Christophe Desterke and Caroline Le Bousse-Kerdiles for their precious help and advice in molecular biology experiments; Delphine Crépin and Jennifer Carrière for excellent technical assistance; and Jan Filipski, Urs Albrecht, Ueli Schübler, and Nicolas Cermakian for helpful and in-depth scientific discussions on these results.

References


Improved Tumor Control through Circadian Clock Induction by Seliciclib, a Cyclin-Dependent Kinase Inhibitor

Ida Iurisci, Elisabeth Filipski, Jens Reinhardt, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/66/22/10720

Cited articles
This article cites 47 articles, 22 of which you can access for free at:
http://cancerres.aacrjournals.org/content/66/22/10720.full.html#ref-list-1

Citing articles
This article has been cited by 11 HighWire-hosted articles. Access the articles at:
/content/66/22/10720.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.