Circadian pharmacology of L-alanosine (SDX-102) in mice

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Abstract

L-Alanosine (SDX-102) exerts its cytotoxicity through inhibition of de novo purine biosynthesis, an effect potentiated by methylthioadenosine phosphorylase (MTAP) deficiency. The relevance of circadian dosing time was investigated for chronotherapeutic optimization of SDX-102. Toxicity was assessed in healthy mice following single (1,150, 1,650, or 1,850 mg/kg/d) or multiple doses (250 or 270 mg/kg/d). Efficacy was tested in mice with P388 leukemia receiving multiple doses (225 or 250 mg/kg/d). SDX-102 was administered at six circadian times 4 hours apart in mice synchronized with 12 hours of light alternating with 12 hours of darkness. MTAP expression was determined in liver, bone marrow, small intestinal mucosa, and P388 cells. Dosing at 19 hours after light onset reduced lethality 5-fold after single administration and 3-fold after multiple doses as compared with worst time (P < 0.001 and P < 0.01, respectively [χ² test]). Neutropenia, lymphopenia, and bone marrow hemorrhagic lesions were significantly less in mice dosed at 19 hours after light onset as compared with 7 hours after light onset. SDX-102 at 7 hours after light onset transiently ablated the 24-hour patterns in body temperature and activity. A circadian rhythm characterized small intestinal MTAP expression with a maximum at 6:30 hours after light onset (P = 0.04). A minor survival improvement was found in MTAP-deficient P388 mice receiving SDX-102 at 7 or 23 hours after light onset as compared with other times (P = 0.03, log-rank test). In conclusion, the therapeutic index of SDX-102 was improved by the delivery of SDX-102 in the mid to late activity span. These results support the concept of chronomodulated infusion of SDX-102 in cancer patients. [Mol Cancer Ther 2006;5(2):337–46]

Introduction

Tolerability and activity of anticancer agents can vary as a function of circadian dosing time in mice, rats, and humans (1–4). These findings have led to the concept of chronotherapeutics, a method aiming at adjustment of treatment to biological rhythms to improve therapeutic index. The dosing time dependency of therapeutic activity results from the circadian regulation of cell division cycle and cellular metabolism (4–6). The circadian timing system is constituted with a central coordination system involving the suprachiasmatic nuclei, the main circadian pacemaker in the hypothalamus, and a molecular clock which equip most cells in the brain and in peripheral tissues. This molecular clock consists of multiple feedback loops involving transcriptional and posttranscriptional processes of 12 specific clock genes. Recent data have emphasized that clock genes control the transcription and/or protein expression of cell cycle–related genes such as wee1, cdce2, cyclin B1, cyclin D1, p53, c-myc, gadd45a, BCL-2, and BAX (7–11). The molecular link between the molecular clock and the cell division cycle and related apoptosis or repair processes make chronopharmacologic approach relevant in the development of cancer drugs targeted at specific molecular pathways.

L-Alanosine (SDX-102) is an amino acid analogue originally derived from *Streptomyces alanosinicus*. It interferes with the de novo synthesis of AMP in both malignant and normal cells. Methylthioadenosine phosphorylase (MTAP), a key enzyme involved in the salvage pathway of purine metabolism, is present in normal tissues but frequently is deleted (deficient) in leukemias, brain tumors, non–small-cell lung cancers, breast cancers, melanomas, pancreatic cancers, and sarcomas (12–17) as a consequence of homologous deletion of the gene located at the p21 loci on chromosome 9 (16). MTA is produced during polyamine synthesis and cleaved to adenine and 5-methylthioribose-1-phosphate by MTAP. The adenine is reconverted to AMP and then to ATP. The deletion of the MTAP gene in many tumors results in the inability of these cancer cells to salvage adenosine; the ATP pools in these cells must be depleted. L-Alanosine, a potent inhibitor of de novo AMP synthesis, has shown selective anticancer activity in *vitro* in MTAP-negative cell lines as compared with MTAP-positive cell lines (17). SDX-102 should deprive such tumor cells (but not normal cells) of de novo capacity to synthesize adenosine (18, 19). Thus, the deficiency of MTAP in tumor cells offers a unique opportunity to develop a selective
therapy sparing normal cells. This selectivity could be enhanced further through the administration of SDX-102 at a specific circadian time that would decrease drug toxicity for normal host cells.

A circadian rhythm characterizes cellular polyamine levels both in rodents and in humans (20, 21). Adenosine plays a role in promoting sleep and the activity of the major metabolic enzymes for adenosine was higher during the active period in brain of rats. This diurnal variation may play a role in the regulation of relationship to sleep and wakefulness across the day (22). Adenosine reportedly regulates the response of the circadian clock to the phase-adjusting effects of light in hamster (23) and contributes to sleep processes in humans, a process under light circadian control (24, 25). Circadian variation has also been reported for purine nucleotide phosphorlase transcription in mouse liver (26). This enzyme is analogous to MTAP in humans and these results suggest that MTAP activity is likely under circadian regulation in humans, which can be successfully exploited in tumor selective therapy with SDX-102.

In patients, SDX-102 dose-limiting toxicities are bone marrow suppression and mucositis (27–30). These side effects are also observed following 5-fluorouracil continuous infusion and have been reduced up to 5-fold through chronomodulated delivery (3). SDX-102 has shown activity against several human tumors in experimental models (31–33) but failed to show activity in phase II clinical trials conducted in the 1980s (27–30). A recent reappraisal of the causes for such discrepancy revealed that the rate of MTAP deletion was <5% in those tumor types that had been selected for these clinical trials (18). This observation has supported additional trials in tumor types with a high frequency of MTAP deletion. A chronotherapeutic approach could enhance SDX-102 tolerability (as maximal doses are limited by mucositis) and efficacy and provide a safer and more effective treatment schedule to the patients with MTAP-deleted tumors.

We examined whether SDX-102 tolerability varied as a function of circadian dosing time in mice receiving single or multiple doses. The relation between chronotolerance and toxicity of this drug to blood cells, bone marrow, and jejunum was investigated. In addition, locomotor activity and body temperature rhythms, two main outputs of the circadian timing system, were assessed. We further evaluated MTAP mRNA transcriptional pattern in liver, bone marrow, and intestinal mucosa as a potential mechanism for the chronotolerance of SDX-102. Finally, the relevance of circadian dosing time on antitumor efficacy was evaluated in P388 leukemia-bearing mice, a MTAP-negative tumor.

Materials and Methods

Animals and Synchronization

Six-week-old, male B6D2F1 mice were purchased from Janvier (Le Genest St Isle, France). They were kept in an autonomous chronobiologic animal facility (Jouan, Saint-Herblain, France; ref. 1). All mice were synchronized with an alternation of 12:12-hour light/darkness, with food and water provided ad libitum throughout all experiments. Experiments were begun after 3 weeks of 12:12-hour light/darkness synchronization. Circadian time was expressed in hours after light onset.

Experiments were carried out in accordance with the guidelines for the welfare of animals in experimental neoplasia approved by the United Kingdom Coordinating Committee on Cancer Research (34).

Tumor Model

P388 lymphocytic leukemia (P388) was obtained from Institut de Recherche Pierre Fabre (Castre, France). Tumors were maintained in DBA female mice (Janvier) and passaged as weekly i.p. implants (35).

Drugs

SDX-102 was provided as a sterile lyophilate by Salmedix, Inc. (San Diego, CA). It was diluted in 0.9% NaCl before each study and injected i.p. (10 mL/kg body weight).

Study Design

Chronotolerance

Three experiments were done in a total of 446 mice. SDX-102 was given as a single dose in experiment 1 and daily for 5 days (days 1–5) in experiments 2 and 3.

In experiment 1, SDX-102 (1,150, 1,650, or 1,850 mg/kg) or 0.9% NaCl were given at one of six circadian times (3, 7, 11, 15, 19, or 23 hours after light onset). There were 5 mice per dosing time for control mice and 12 or 13 mice per dose level and per dosing time for SDX-102-treated mice.

In experiment 2, SDX-102 (275 mg/kg/d) was injected daily for 5 days at 3, 7, 11, 15, 19, or 23 hours after light onset. There were 13 to 14 mice per dosing time.

In experiment 3, SDX-102 (250 mg/kg/d) or 0.9% NaCl was injected daily for 5 days at 7 or 19 hours after light onset (n = 57 per dosing time). Survival and body weight change were monitored in 30 mice receiving SDX-102 at either dosing time (15 mice per time point). Additionally, 4 controls and 10 mice receiving SDX-102 at 7 or 9 hours after light onset were used for blood cell counts and histologic study in bone marrow and jejunum.

Lymphocyte and Neutrophil Counts. Blood was sampled 7, 10, or 12 days after treatment onset at 7 or 19 hours after light onset for lymphocyte and neutrophil counts (Cell-Dyn, Abbott Diagnostics, Rungis, France).

Histologic Lesions. Femoral bone marrow and jejunum were obtained at 7 or 19 hours after light onset immediately after sacrifice and fixed into 4% paraformaldehyde. Twenty-four hours later, the samples were dehydrated and embedded into paraffin. Sections were made and stained with hemalum-erythrosine-safran. Each slide was examined by the same histopathologist and lesions were graded in a blind manner. Jejunum lesions were scored as 1 for each of the following items: surface epithelial cells, villi structure, and crypt gland cells. The sum of all three scores was computed as being a toxicity grade, ranging from 0 (normal) to 3 (alteration for each item). Bone
marrow lesions were graded separately for each of the following criteria: cellularity from 0 (normal) to 3 (severe aplasia) and extent of hemorrhagic lesions in medullar tissue from 0 (normal) to 3 (severe).

**SDX-102 Effects on Two Circadian Clock Outputs**

In experiment 4, mice were implanted i.p. with a telemetric temperature and activity sensor (PhysioTel, TA10TA-F20, Data Sciences, St. Paul, MN). I.p. temperature and locomotor activity were automatically monitored every 10 minutes using the Dataquest A.R.T. analysis system (Data Sciences) during the entire study.

Animals were singly housed and synchronized with 12:12-hour light/darkness for 2 weeks, then exposed to constant darkness on the day of treatment onset until the end of the experiment. Treatment consisted of the daily administration of SDX-102 (225 mg/kg/d, n = 3) or 0.9% NaCl (n = 3) at 14:00, subjective 7 hours after light onset for 4 days (days 1–4). After treatment, all animals were exposed to constant darkness for another 10 days. Rhythm monitoring in mice kept in constant darkness allowed to identify SDX-102 effects on circadian clock outputs without the regular resetting by recurring 12-hour exposures to light.

**Tissue Sampling for MTAP Gene Expression**

In experiment 5, mice were sacrificed at 3, 7, 11, 15, 19, or 23 hours after light onset (n = 6 per time) following synchronization. The animals were exposed to constant darkness during the preceding 24-hour sampling. This procedure avoids any masking effect of light itself on the endogenous circadian rhythm (36).

Liver, small intestine, and two femurs and tibias were sampled immediately from each mouse. Liver was transferred to RNAlater (Ambion Ltd., Cambridgeshire, United Kingdom) and stored at −80°C until RNA extraction.

Bone marrow cells were collected by repeatedly flushing the femurs and tibias with PBS through a 26-G needle, then suspended in RNAlater and stored at −80°C until RNA extraction.

Intestine was sectioned from 0.5 cm below the stomach to 1 cm above the cecum. The intestinal lumen was washed with PBS, then cut open longitudinally. Intestinal epithelium was harvested by lightly scraping the surface, resuspended in PBS, and RNAlater was added. The cells were stored at −80°C until RNA extraction.

**Quantitative Real-time PCR**

Total RNA was extracted from tissues using the RNAqueous-4PCR method (Ambion, Inc., Austin, TX). Extracted RNA samples were treated with DNase I and stored at −80°C until use. Approximately 1 μg of total RNA was used in a reverse transcriptase PCR reaction with oligo(dT)20 primers to generate total cDNA (Invitrogen, San Diego, CA).

Quantitative real-time PCR was done using the iCycler iQ Real-time PCR Detection System (Bio-Rad, Hercules, CA). The MTAP primers were MTAP-F (5’-GTTGCCAAGCGCTGATG-3’) and MTAP-R (5’-TGTGTGCTGTTG-3’). For sample normalization, a reference control gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was amplified using the primers GAPDH3-F (5’-TCACCCACATGGAGAAGGC-3’) and GAPDH3-R (5’-GCTAAGCAGTTGGTGCTGCA-3’). The quantitative PCR analysis was done at 95°C for 3 minutes followed by 50 amplification cycles at 95°C (10 seconds), 56°C (30 seconds), and 72°C (30 seconds). A melt curve analysis was done initially at 55°C with a heating rate of 0.5°C/s using a continuous fluorescence measurement.

Expression in liver was arbitrarily chosen as a calibrator. The fold change in MTAP gene expression normalized to the GAPDH reference gene and relative to the calibrator was computed and the data were expressed as percent of 24-hour mean value.

**MTAP Deletion in P388**

MTAP status of the P388 line was evaluated by both PCR and the aforementioned quantitative PCR method, with mouse spleen and/or liver as a positive reference. For genomic PCR analysis, two sets of MTAP primers for mouse were designed (Salmedix): M-MTAP (sense, 5’-GCTGCTGACACAAGAAAG-3’; antisense, 5’-TATACGG-GAAACACGGCACA-3’) and M-MTAP_1 (sense, 5’-CAG-CGCTAAAGGAGGCAATAC-3’; antisense, 5’-TATACGG-GAAACACCCGCAACA-3’). GAPDH served as the housekeeping and positive control primer set. Following isolation using the DNeasy Tissue Kit (Qiagen, Valencia, CA), 2 μg of DNA were added to total reaction volume of 25 μL, containing 0.5 μL of each 10 μmol/L primer and 12.5 μL of AccuPrime Supermix I (Invitrogen). Using a touchdown PCR method, samples were subjected to 10 cycles of incremental decreases in the annealing temperature at 1°C per round starting at an initial temperature of 60°C. This was followed by an additional 20 cycles of amplification at an annealing temperature of 50°C. Once complete, a 10-μL volume of the products was resolved on a 2% agarose gel by electrophoresis.

**Chronoefficacy**

Three experiments were done involving a total of 333 mice. P388 cells (10⁶) were inoculated on day 0 between 10:00 and 11:30 because previous experiment had shown the lack of influence of the circadian time of P388 inoculation on mouse survival (35). Multiple doses of SDX-102 were subsequently administered at 3, 7, 11, 15, 19, or 23 hours after light onset beginning 24 hours following tumor inoculation, with survival as end point.

In experiments 6 and 7, SDX-102 (250 mg/kg/d) or 0.9% NaCl was injected daily for 5 days (days 1–5 schedule) and on days 1 to 3, days 8 to 10, and days 15 to 17, respectively. For each experiment, there were 3 control mice and 13 to 16 SDX-102-treated mice at each dosing time.

In experiment 8, SDX-102 (225 mg/kg/d) or 0.9% NaCl was administered on days 1 to 4, days 8 to 11, and days 15 to 18. There were 4 controls and 17 SDX-102-treated mice per time point.

**Statistical Analysis**

Mean ± SE was calculated for each variable. Body weight change was analyzed by one-way or two-way ANOVA. The differences were validated by Kruskal-Wallis test for nonparametric data. Survival curves were drawn according
to Kaplan-Meier and differences were analyzed by log-rank test. This analysis was done both on all data and without including toxic deaths. Survival rate at 50% overall mortality was analyzed as a function of group by \( \chi^2 \) test.

Dosing time dependencies were further analyzed by cosinor (37) with periods of 24 and 12 hours for body weight change and survival. This method computes the mesor (midline estimating statistic of rhythm or rhythm-adjusted mean), the double amplitude (difference between maximum and minimum in fitted cosine function), and acrophase (time of maximum in fitted cosine function, with light onset as a phase reference). The 95% confidence limits of these variables were calculated. \( P < 0.05 \) was required for statistical significance.

Temperature (°C) and activity data (arbitrary units) were analyzed with power spectrum analysis done with Mathematica (Wolfram Research, Inc., Champaign, IL) to determine the dominant period. The period that corresponded to the highest amplitude was considered as the dominant one. In experiment 5, the analysis was done on time series of 6 days (before treatment in 12:12-hour light/darkness), 4 days (during treatment in constant darkness), and 10 days (after treatment in constant darkness).

**Results**

**Chronotolerance**

**Single Dose**

In experiment 1, 1 of 77 mice (1.3%) receiving 1,150 mg/kg SDX-102 died from toxicity as compared with 41 of 79 (52.0%) receiving 1,650 mg/kg or 53 of 72 (74.0%) given 1,850 mg/kg (\( \chi^2 = 85.3, P < 0.001 \)). These toxic-related deaths occurred between 2 and 6 days following dosing. Moreover, a clear circadian dependency of SDX-102 toxicity was found as shown by significantly different survival curves, with best survival in mice given SDX-102 at 19 hours after light onset (Fig. 1A). Irrespective of SDX-102 dose, lethality was encountered in 4 (16%) or 7 mice (28%) treated at 19 or 23 hours after light onset, respectively, as compared with 19 mice (76%) treated at 15 hours after light onset (\( \chi^2 = 23.1, P < 0.001 \)). A 24-hour rhythm in survival time was statistically validated by cosinor analysis with an acrophase located at 22:50 hours after light onset (Fig. 2).

Irrespective of dosing time, a dose-response was seen in weight loss nadir that was reached 1 or 2 days after SDX-102 injection (1,150 mg/kg, 8.7 ± 0.3%; 1,650 mg/kg, 9.5 ± 0.5%; 1,850 mg/kg, 10.9 ± 0.5%; \( P = 0.001, \) ANOVA). The relation between dose and body weight loss was significantly steeper following dosing at 3, 7, 19, or 23 hours after light onset as compared with SDX-102 treatment at 11 or 15 hours after light onset (\( P = 0.02; \) data not shown). Complete recovery was achieved 3 days after SDX-102 dosing in mice treated at 15 hours after light onset whereas this was not the case for the other dosing time groups. According to cosinor analysis, the dosing time estimated to produce least body weight loss and fastest recovery was located at 22:50 hours after light onset (Fig. 2).

**Multiple Doses**

**Survival and Body Weight Loss.** Following five daily doses, survival rate was highest in the mice treated at 19 hours after light onset as compared with those given the drug at 3, 7, or 11 hours after light onset (Fig. 1B and C). In experiment 2, a 24-hour rhythm in survival time was statistically validated by cosinor analysis, with an acrophase at 18:55 hours after light onset (Fig. 2). Body weight gradually decreased during treatment, reaching a nadir of \(-27.4 ± 0.6\%\) 2 days after the last dose irrespective of dosing time. Body weight recovery was complete 12 days after treatment onset in the mice treated at 23 hours after light onset but not for the other groups. Two-way ANOVA further validated a significant difference according to dosing time and experimental day, with an

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Figure 1. Survival curves of healthy mice receiving SDX-102 injection(s) (1) at one of six circadian times in experiments 1 and 2 or at one of two dosing times in experiment 3. **A**, single dose (experiment 1); **B**, multiple doses (experiment 2: 275 mg/kg/d on days 1 – 5); **C**, multiple doses (experiment 3: 250 mg/kg/d on days 1 – 5). The survival differed according to SDX-102 dosing time, with longer one following single or multiple administration at 19 h after light onset (HALO): **A**, \( P = 0.01 \); **B**, \( P = 0.05 \); **C**, \( P = 0.1 \) (log-rank test).
interaction term ($P \leq 0.02$). A circadian rhythm in body weight change on day 12 was validated with cosinor analysis, with an acrophase at 23:55 hours after light onset (Fig. 2).

In experiment 3, body weight loss nadir ($-28.5 \pm 0.6\%$) was reached 7 days after treatment onset. Body weight recovery was complete 15 days after treatment onset in the mice treated at 19 hours after light onset but not at 7 hours after light onset. Two-way ANOVA further validated a significant difference according to dosing time and experimental day ($P < 0.001$) without any interactions.

Overall, the survival rate of the mice was best following treatment at 19 hours after light onset as compared with 7 hours after light onset (85.7\% versus 53.6\%; $\chi^2 = 6.8$, $P < 0.01$).

**Hematologic Toxicity.** In controls, mean counts of circulating leukocyte, lymphocyte, and neutrophil were $\approx 50\%$ larger at 7 hours after light onset as compared with 19 hours after light onset as a result of their well-known circadian rhythm (38).

As compared with controls, lymphocyte and neutrophil counts dropped by $98.7 \pm 0.2\%$ and $79.1 \pm 5.4\%$, respectively, 7 days after SDX-102 treatment onset without significant difference according to dosing time. However, complete recovery was achieved 3 days later in the mice treated at 19 hours after light onset but not for the group treated at 7 hours after light onset (Fig. 3).

**Intestinal and Bone Marrow Toxicities.** Most extensive jejunum and bone marrow lesions were encountered 2 days after the fifth dose of SDX-102. These organs had completely recovered 3 days later regardless of dosing time.

On the day of maximal toxicity, all the treated mice had pathologic changes in gut epithelium, irrespective of dosing time. However, 5 of 10 (50\%) mice receiving SDX-102 at 19 hours after light onset had villi abrasion and crypt gland cell lesions compared with 1 of 7 (14\%) treated at 7 hours after light onset (Fig. 4). Thus, the extent of jejunum damage appeared to be more severe in the mice treated at 19 hours after light onset (mean histologic grade $\pm SE$, 2.6 $\pm 0.3$) as compared with those treated at 7 hours after light onset (1.4 $\pm 0.2$; $P = 0.02$, Kruskal-Wallis).

Bone marrow alterations mostly consisted of hypoplasia, aplasia, and hemorrhagic lesions. Of 14 mice assessable for bone marrow damage, 1 displayed slight aplasia (grade 1) and 6 displayed moderate aplasia (grade 2) at each dosing time (1.9 $\pm 0.1$). However, histologic scores for hemorrhagic lesions were more severe at 7 hours after light onset ($2.7 \pm 0.3$; 6 of 7 grade 3) as compared with 19 hours after light onset (1.6 $\pm 0.3$; 1 of 7 grade 3; $P = 0.02$, Kruskal-Wallis; Fig. 4).
SDX-102 Effects on Two Circadian Clock Outputs

All the mice displayed stable 24-hour rhythms in body temperature and locomotor activity in 12:12-hour light/darkness. Following exposure of the mice to constant darkness on the day of treatment onset, no obvious effect of daily saline injection was noticed, but with a shorter period. Conversely, daily SDX-102 at subjective 7 hours after light onset suppressed both circadian rhythms in all three mice during treatment. Recovery of near normal circadian rhythms was apparent ≈4 days after treatment withdrawal.

Spectral analysis showed that in 12:12-hour light/darkness, the mean dominant periods of temperature and activity rhythms were 23.8 ± 0.04 and 23.6 ± 0.2 hours, respectively. In constant darkness, free-running body temperature rhythm had a stable period of 23.6 ± 0.5 hours during saline treatment and after withdrawal. SDX-102 delivery completely ablated both rhythms. After withdrawal, circadian rhythms recovered with a period of ≈23.5 hours and amplitudes similar to those found in controls.

MTAP Gene Expression

The mean relative abundance of MTAP mRNA expression was 0.94 ± 0.04 in liver, 0.48 ± 0.04 in bone marrow, and 0.02 ± 0.002 in small intestinal mucosa (P < 0.001). No temporal change in MTAP expression was statistically significant in liver and bone marrow. Conversely, MTAP expression varied over the 24 hours in small intestine. It was highest during the light span and lowest during the darkness. The respective peak-trough difference was ≈2-fold (Fig. 5). A sinusoidal 24-hour rhythm of MTAP expression in small intestine was further validated with cosinor, with an acrophase located at 6:30 hours after light onset (95% confidence limits: 1:20 to 11:30; P = 0.04).

Antitumor Efficacy

In the P388 cell line we have used, we confirmed the deletion of the genomic transcript of MTAP by PCR (Fig. 6). In each of the three antitumor efficacy experiment, untreated control mice died 8 to 12 days after P388 inoculation.

Of 93 mice receiving daily SDX-102 (250 mg/kg/d) for 5 days in experiment 6, 28 (30.1%) died from drug...
toxicity between 7 and 10 days after tumor inoculation without any sign of ascites. Toxic deaths mostly occurred following SDX-102 at 7 or 23 hours after light onset [7 of 16 mice (43.8%) or 8 of 16 (50%), respectively]. Conversely, the incidence of toxic deaths was least following treatment at 15 hours after light onset (2 of 16, 12.5%) or 19 hours after light onset (2 of 14, 14.3%; $\chi^2 = 10.4, P = 0.06$). The 65 treated mice which did not succumb to drug toxicity died from tumor progression with ascites 18 to 21 days after P388 inoculation. Median survival time was 10 days in controls and 19 days in treated mice ($P = 0.002$, log-rank test; Fig. 7A). No statistically significant difference in survival was observed as a function of SDX-102 dosing time, an outcome possibly biased by drug toxicity at this dosing frequency.

Of 78 mice given SDX-102 (250 mg/kg/d) daily for 3 days every week in experiment 7, a single animal treated at 15 hours after light onset died from drug toxicity 16 days after tumor inoculation (1.3%). The remaining treated mice died from tumor progression 20 to 34 days after tumor inoculation. Median survival time was 10 days in controls and 22 days in treated mice ($P < 0.001$, log-rank test; Fig. 7A). A 12-hour rhythmic component in survival time was statistically validated with cosinor analysis, with 7:10 and 19:10 estimated as the dosing times achieving best efficacy.

No lethality resulted from SDX-102 daily treatment (225 mg/kg/d) for 4 days every week in experiment 8. Treated mice died from tumor progression 21 to 34 days after P388 inoculation. Median survival time was 10 days in controls and 28 days in treated mice ($P < 0.001$, log-rank test). Thus, this treatment schedule was the most active one (Fig. 7A). The survival curves of mice receiving this treatment schedule varied as a function of dosing time ($P = 0.03$, log-rank test) with best survival in mice given SDX-102 at 7 or at 23 hours after light onset (Fig. 7B).

Overall, the dose-adjusted survival of SDX-treated mice on both chronic weekly treatment (experiments 7 and 8) significantly differed as a function of dosing time ($P = 0.01$, log-rank test).

**Discussion**

The toxicity experiments have unambiguously shown that SDX-102 lethality varied largely as a function of circadian dosing time. Least lethality was found following single or multiple doses of SDX-102 in the second half of the activity span. The cosinor-estimated acrophases of survival time, corresponding to best tolerability, were similar with either dose schedule, as they respectively occurred at 22:50 and 18:55 hours after light onset with overlapping 95% confidence limits (Fig. 2). Conversely, the mice given a single high dose of the drug lost the least weight following dosing at 11:35 hours after light onset (acrophase) whereas no time-related difference in body...
weight nadir was found in the mice given multiple doses of SDX-102. These discrepancies might relate to a complex pattern of the jejunal toxicity of the drug, as suggested by the histologic findings. However, recovery from body weight loss was significantly faster in the mice receiving multiple doses of SDX-102 at 23:35 hours after light onset (acrophase), a finding consistent with the above survival data (Fig. 2). Also consistent with these observations were the facts that both bone marrow hemorrhagic lesions at maximal toxicity were least and recovery from leuconeutropenia was fastest in the mice treated at 19 hours after light onset as compared with 7 hours after light onset. In contrast, jejunal lesions appeared as more severe on day 7 in the mice treated at 19 hours after light onset, with complete recovery 3 days later. Possibly the toxicokinetics of bone marrow and jejunal lesions would require more frequent sampling including during the treatment span itself. Therefore, where the administration of single or multiple doses of SDX-102 is contemplated, the optimal time to be recommended for optimizing tolerability is the second half of the activity span.

We further showed that daily SDX-102 (225 mg/kg/d) for 4 days resulted in a transient suppression of the circadian rhythms in body temperature and locomotor activity, two circadian clock outputs in all the mice. Similar results have been obtained in mice receiving vinorelbine or in rats receiving a single dose of cisplatin (39, 40). The mechanisms through which SDX-102 altered both circadian rhythms are unknown. Bilateral suprachiasmatic nuclear destruction permanently suppressed the activity rhythm in all mice and the temperature rhythm in 85% of them (41). The present findings suggest that SDX-102 may alter transiently the coordination of circadian clock function directly or via an action on efferent pathways from suprachiasmatic nuclei and/ or peripheral oscillators. SDX-102-induced circadian dysfunction may contribute to the toxicokinetics of this and possibly other coadministered anticancer drugs (42).

To gain further understanding on chronotoxicity and chronoefficacy of SDX-102, MTAP mRNA expression was investigated in liver, small intestinal epithelium, and bone marrow, the target organs along the 24-hour time scale. GAPDH was used as a reference control gene because its expression was reported to be constant throughout the 24 hours (43–45). In separate studies, we used 36B4 as time invariant gene and found a circadian expression of clock gene Per2 similar to that reported using GAPDH as an internal control (45, 46). Taken together, these data support the use of GAPDH as an internal control for circadian expression studies. We found that MTAP expression was expressed 56- and 23-fold less in small intestine as compared with liver and bone marrow, respectively. The lower level of expression in small intestinal epithelium correlates well with the mucosal toxicity of SDX-102 in patients (27–30). A circadian rhythm of MTAP expression in intestinal epithelium was statistically significant with a maximum in the middle of light span. This latter time corresponded to the circadian time of least jejunal damage induced by SDX-102 observed in chronotolerance study.

The relation between the circadian rhythm in drug tolerability and antitumor efficacy constitutes an important issue for the development of chronotherapeutic schedules with SDX-102. The P388 leukemia model was chosen because it displayed a deficiency of MTAP, a condition known to favor the antitumor activity of SDX-102. Indeed, a single dose of SDX-102 to P388 leukemic mice completely stopped DNA synthesis in the P388 cells within 4 hours and such inhibition was sustained for 24 hours. These results indicated the consistency of in vitro and in vivo expression of MTAP as well as its consequence for the antitumor activity of SDX-102 in this model (47). In the P388 cell line we have used, we confirmed the deletion of the genomic transcript of MTAP by PCR and the absence of a significant mRNA transcript by quantitative PCR. The lack of a functional MTAP was also evaluated indirectly by an enzymatic assay measuring ATP levels. Our experiments in P388-bearing mice showed that lethality also displayed a circadian rhythm following multiple doses of SDX-102. Greater drug-associated mortality was encountered in the mice treated at 7 or 23 hours after light onset as compared with 15 or 19 hours after light onset. Such variation was similar to that found in the chronotolerability experiments. These findings suggest that the presence of an early-stage, yet fast-growing, tumor produced minimal, if any, alteration of the rhythm in SDX-102 tolerability although tumor bearing mice seem to be somewhat more sensitive to SDX-102 toxicity than are non-tumor-bearing animals. This confirms similar reports with 1-β-D-arabinofuranosylcytosine, docetaxel, and vinorelbine (2, 35, 48).

In the study in which SDX-102 (250 mg/kg/d) was given on days 1 to 5, median survival of leukemic mice doubled following SDX-102 treatment as compared with controls. Yet, no statistically significant difference in survival was observed as a function of dosing time on this intense schedule. However, the administration of two nontoxic weekly schedules enhanced overall survival. The dosing time–related differences in efficacy were statistically significant, yet of small magnitude, in this rapidly proliferating tumor model. The most active treatment corresponded to SDX-102 dosing near the middle of light span (mid-rest) or near the middle of the dark span (mid-activity). Such bimodal pattern in the antitumor efficacy of SDX-102 may reflect a deregulated circadian rhythm in another metabolic pathway relevant for SDX-102 activity in P388 cells with MTAP deficiency. This latter time corresponded to the circadian time of least toxicities of SDX-102 observed in previous experiments. Such coincidence between the circadian times associated with best efficacy and best tolerability was also observed for single agent doxorubicin, docetaxel, or vinorelbine and for doxorubicin-cisplatin, docetaxel-doxorubicin, or irinotecan-oxaliplatin combination (1, 2, 35, 49, 50). In conclusion, the therapeutic index of SDX-102 was improved by the delivery
of SDX-102 in 19 hours after light onset in MTAP-deficient tumors. At the clinic, this optimal schedule would correspond to the delivery of SDX-102 in the mid to late activity span, an issue to be further investigated in cancer patients.

Acknowledgments
We thank Brian Crain, Heather Bendall, and Michael Jason Virata of Salmedix for their excellent work in MTAP analysis.

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Molecular Cancer Therapeutics

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Mol Cancer Ther 2006;5:337-346.

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