

# Chronotherapy and Chronotoxicity of the Cyclooxygenase-2 Inhibitor, Celecoxib, in Athymic Mice Bearing Human Breast Cancer Xenografts<sup>1</sup>

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## ABSTRACT

**Purpose:** Inhibition of the enzyme cyclooxygenase with celecoxib is cytotoxic in a variety of solid tumor cell lines. Previous work has shown that by charting circadian rhythms, it has been possible to find optimal times to deliver a dose of drug, such that it is most efficacious in killing cancer cells and least harmful to normal tissues. Therefore, we examined the time dependence of toxicity (chronotoxicity) and of antitumor effects (chronotherapy) of celecoxib to determine optimal time of day for dosing with respect to light-dark cycles.

**Experimental Design:** Celecoxib was administered i.p. for 10 days (5 days on, 2 days off, 5 days on) to nude mice bearing s.c. breast xenografts. Body weight, peripheral blood cells, clinical chemistry, and tumor growth were monitored.

**Results:** The highest tolerance (100% survival) was found at 7 HALO and the least occurred at 17 h after light onset (HALO; 10% survival). Chronotherapy at a 20-mg/kg dose varied between the seven HALO evaluated and between the three breast tumors (MCF-7, ZR-75-30, and MDA-MB-468) studied. When the maximum tolerated dose (MTD) of celecoxib was optimized for each HALO, we found that at 7–10 HALO, the MTD was 25 mg/kg, whereas at 17–20 HALO; the MTD was only 10 mg/kg. Tumor regression was observed when dosing was done at 23 HALO to 7 HALO (5 a.m. to 1 p.m.), whereas no therapeutic response was observed when dosing was done at 10–13 HALO (4 p.m. to 7 p.m.), and rapid tumor growth was noted when dosing was done at 17 HALO (11 p.m.).

**Conclusions:** Tumor growth response to the MTD at each HALO revealed that there was no clear relationship

between dose administered and therapeutic response. COX-2 expression was not able to explain either the chronotherapy or the chronotoxicity results obtained.

## INTRODUCTION

Current breast cancer incidence and mortality rates (>184,000 and >43,000 cases/year, respectively; Ref. 1) highlight the need to explore alternative therapeutic strategies. One cellular pathway that appears to have a significant impact on breast carcinogenesis as well as the growth of established breast tumors is the COX<sup>3</sup> (COX-2)/prostaglandin-mediated pathway (2–4). COX-2 expression contributes to tumorigenic potential of epithelial cells by increasing adhesion to extracellular matrix and making them resistant to apoptosis (5). COX-2 also plays a vital role in regulation of angiogenesis of neoplastic cells (6). COX-2 is a favorable and promising therapeutic target for prevention and/or therapy of various solid tumors including breast cancer (3, 4, 7–9). It catalyzes the rate-limiting step in the formation of an array of prostaglandins from arachidonic acid. COX inhibitors have been shown to have effects for both the prevention and treatment of experimental breast cancer (4, 10, 11). The role of fatty acids and prostaglandins on the growth of experimental breast cancer is complex, with some types promoting and others inhibiting growth *in vitro* or *in vivo* (12). There is some evidence to suggest that accumulation of arachidonic acid is growth inhibitory and that breast cancer cell line growth is inhibited secondary to increases in lipoyxygenase pathway end products. Thus, cyclooxygenase inhibition may lead to accumulation of arachidonic acid or lipoyxygenase products, which may contribute to the inhibitory effects of COX inhibitors in cancer. In addition to the direct antitumor effects of these drugs, indirect growth control resulting from an antiangiogenic effect also occurs (13–15).

Immunohistological analysis of human breast cancer specimens as well as derivative cell lines has shown a preponderance of expression of the COX-2 enzyme over COX-1 (9). COX-2 is not expressed constitutively by normal cell types but is found to be expressed constitutively in cancerous tissues (9, 16). Thus, selective COX-2 inhibitors would be predicted to have similar effects as first- and second-generation, nonspecific COX inhibitors (*i.e.*, nonsteroidal anti-inflammatory drugs) with a more favorable toxicity profile (17). Indeed, *in vivo* and *in vitro* data confirm the suppressive effects of COX-2-selective inhibitors on human breast cancer models. Progress in the development of

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<sup>3</sup> The abbreviations used are: COX, cyclooxygenase; MTD, maximum tolerated dose; ER, estrogen receptor; HALO, hours after light onset; RT-PCR, reverse transcription-PCR; AST, aspartate aminotransferase; BUN, blood urea nitrogen.

pharmacological agents that target COX-2 selectively has led to the registration of next-generation drugs (e.g., celecoxib and rofecoxib) with well-known safety profiles. There is much interest in using these agents both alone and in combination with chemotherapy, radiotherapy, or other anticancer modalities. This study has tested the hypothesis that COX-2-selective inhibitors can be further optimized as novel therapeutic agents for treatment of breast cancer using chronobiological principles. By optimizing the time of day for dosing, the MTD can be adjusted, possibly allowing for a greater therapeutic effect with minimal toxic side effects.

Differences in rates of cell cycle progression as a function of time of day account, in part, for the circadian variation in sensitivity of tissues to cytotoxic drugs (chronotoxicity/chronotolerance; Ref. 18). Because cell cycle-dependent differences in expression of COX-2 have been demonstrated recently (19);  $G_1 > S > G_2$ -M), the possibility for chronotherapy and chronotoxicity effects is even greater. These effects, taken together with the chronopharmacology of anticancer drugs (e.g., circadian changes in pharmacokinetics and pharmacodynamics), can account for the diurnal variability of both tolerance and antitumor efficacy (20–23). Tolerability of ~30 anticancer drugs can vary by 50% or more according to circadian rhythms in laboratory animals. Indeed, therapeutic outcome has improved by up to three hundred % by treating rodents at the “right” time under controlled laboratory conditions (24). Clinical trials have confirmed the overall ability to deliver higher doses of chemotherapy with improvement in clinical outcomes by incorporating chronobiological principles (21, 22). This report addresses the issue of chronomodulation of celecoxib as a means of augmenting therapeutic efficacy and/or minimizing toxicity. Celecoxib has a therapeutic effect and has been introduced for therapy of a premalignant condition, familial adenomatous polyposis, which predisposes afflicted patients to the development of invasive colon carcinoma. This drug has efficacy in this disorder as well as other inflammatory/arthritis conditions on a twice-a-day schedule. To our knowledge, this is the first attempt to optimize the therapeutic index of this class of pharmacological agents using a chronobiological approach.

## MATERIALS AND METHODS

**Human Breast Cancer Xenograft Model System and Therapy Experiments.** s.c. tumors were established using the MCF-7 (ER+, MDR-, p53 wt), ZR-75-30 (ER-, MDR+, p53 mut) and MDA-MB-468 (ER-, MDR-, p53 null) cell lines. Three lines were selected for these studies in an effort to develop principles that applied to breast tumors with different genetic characteristics. These lines differ in COX-2 expression *in vitro* (ZR75-30 is the only one that is positive). For all three cell lines, athymic female nude mice (Taconic; 5–6 weeks of age or a minimum starting weight of 18 g) were used. Animal care was provided in accordance with institutional guidelines. Two days prior to MCF-7 tumor cell implantation, mice were estrogenized by i.m. injection of Depo-estradiol (30  $\mu$ l of a 1:3 dilution of stock solution; Florida Infusion, Inc., Palm Harbor, FL). Cultured MCF-7,  $15 \times 10^6$  cells/mouse, was suspended in 0.10 ml of Matrigel stock solution (Collaborative Biomedical Products, Bedford, MA), 0.05 ml of a 2% sterile gelatin solution

Table 1 Time of day versus HALO (based on room lights on at 6 a.m. and room lights off at 6 p.m.)

Time	HALO
9 a.m.	3
1 p.m.	7
4 p.m.	10
7 p.m.	13
11 p.m.	17
2 a.m.	20
5 a.m.	23

(Sigma Chemical Co.-Aldrich, St. Louis, MO), and 0.15 ml of RPMI 1640 with 10% FBS (final injection volume, 0.3 ml/mouse) for adequate tumor take. ZR-75-30 and MDA-MB-468 implantation require only  $5\text{--}8 \times 10^6$  cells/mouse and does not require estrogen pretreatment of the mice. Once tumors reached about 3–4 mm in largest diameter, the tumor-bearing mice were segregated into equivalent groups of 8–10 mice for the therapy experiments.

**Chronobiological Experiments.** Animals were placed on one of three 12/12 light/dark schedules as described previously (25, 26) for 3 weeks prior to the start of all studies to allow for standardization of chronobiological rhythms. Room temperature was kept at  $22 \pm 2^\circ\text{C}$ . Groups of synchronized mice were dosed at each of seven HALO points (3, 7, 10, 13, 17, 20, and 23 HALO). Table 1 provides the relationship between the dosing time in HALO and the actual time of day. Using HALO for time measurement instead of an absolute time of day allows studies between different laboratories with independent light/dark cycles in their animal facility to be compared. By segregating animals into three rooms with different light/dark cycles, as depicted in our previously published chronobiological studies (25, 26), all animal work could actually be completed between 8 a.m. and 4 p.m. on any given day.

**Tumor Therapy and Toxicity Studies.** Celecoxib (Pharmacia, Peapack, NJ; 100- or 200-mg capsules) was prepared in DMSO in a rocking water bath at  $40^\circ\text{C}$  overnight. The material was centrifuged at 1800 rpm  $\times$  10 min. The resulting supernatant was collected and sterile filtered (0.2  $\mu\text{m}$ ). The concentration for the first set of studies was adjusted so that mice were injected i.p. (alternate sides daily) with 25  $\mu$ l of drug or vehicle, i.e., for a 20 mg/kg dose, a 20-g mouse received 25  $\mu$ l of a 16-mg/ml celecoxib solution. Mice were dosed for 5 days, followed by 2 days off to rest and reduce possible toxicity, followed by another 5 days of dosing. The 2 days off was designed to increase the likelihood that the mice will tolerate higher doses. Because all mice in all treatment groups shared the same dose scheduling, the 2-day break should not impact the overall pattern of the results. Groups of 8–10 mice were used for therapy experiments. Animals were weighed prior to therapy and on days 7, 10, and 14 after the start of celecoxib therapy. The percentage of change in body weight at each time point was determined. Tumor size was monitored weekly by three-dimensional caliper measurements, and tumor volume ( $L \times W \times D$ ) was calculated. Actual tumor size in  $\text{cm}^3$ , change in size from time of treatment, and growth inhibition was determined:

$$\text{Tumor size} = \frac{\text{size}_{\text{untreated}} - \text{size}_{\text{treated}}}{\text{size}_{\text{untreated}}} \times 100$$

The significance in the differences in area under the curve at defined times after therapy between two treatment groups was evaluated by the Student's *t* test. Renal (BUN and uric acid) and hepatic (AST and bilirubin) profiles were performed on serum samples at specified HALO every 3 days after therapy with an ATAC6000 Clinical Chemistry Analyzer, using standards and normal positive and negative controls with every run. The mean  $\pm$  SD of each group was recorded ( $n = 4-6$  mice/group). WBC counts were determined as described previously (27) on day 0 and day 10 after celecoxib therapy, and the percentage of change for each animal was recorded. Maximal dose-finding studies were done for each HALO with three doses of celecoxib at 5 mg/kg intervals. The MTD is defined as the highest dose that can be given while still resulting in zero lethality and <20% body weight loss.

**Quantitative RT-PCR.** Cultured cells ( $10^7$ ) were harvested and washed twice with PBS + 0.02%  $\text{NaN}_3$ . Frozen tumor specimens (200  $\mu\text{g}$ ) were collected. The starting template material for RT-PCR was total RNA that was solubilized with a guanidine isothiocyanate-based buffer (Tri-Reagent; Sigma Chemical Co.-Aldrich). RNA was isolated according to a modification of the method of Chomczynski and Mackey (28). Total RNA (5  $\mu\text{g}$ ) was used as the template for cDNA synthesis, using the First Strand kit of Novagen (Madison, WI) according to the manufacturer's instructions, with 25% of the resulting cDNA product being used as the template for each PCR reaction. Primers [for forward sequence, huCOX-2 accession no. UO4636, nucleotides 1821-1840 (gene sequence), ATC CTT GCT GTT CCC ACC CA; and for reverse sequence, huCOX-2 accession no. UO4636, nucleotides 2976-2995 (gene sequence), CTT TGA CAC CCA AGG GAG TC] and 200  $\mu\text{M}$  deoxynucleotide triphosphates were added at standard concentrations, 0.5 and 200  $\mu\text{M}$ , respectively, along with 2 mM  $\text{MgCl}_2$ . One unit of RedTaq thermostable DNA polymerase (Sigma Chemical Co.-Aldrich) was added to each tube, and PCR was carried out under the following initial conditions: annealing temperature of 53°C  $\times$  45 s, extension at 72°C  $\times$  60 s, and denaturation at 94°C  $\times$  30 s  $\times$  50 cycles (initial denaturation is 94°C). PCR reaction products were separated on a 6% TBE (45 mM Tris-borate mM EDTA) polyacrylamide gel containing SYNR Green-I (Sigma Chemical Co.-Aldrich) at a 1:10,000 dilution and visualized on a UV transilluminator and photographed with a Polaroid camera.

**Western Blotting.** Approximately 0.05-0.1 g of tumor xenografts (dissected into <1-mm pieces) was solubilized with a buffer consisting of 1.5% Zwittergent 3-12 in 0.05 M Tris-HCl (pH 8.0) containing 0.15 M NaCl, 2 mM phenylmethylsulfonyl fluoride, and 1  $\times$  protease inhibitor mixture (Boehringer Mannheim, Mannheim, Germany). After solubilization at 4°C for 60 min, nuclei and debris were pelleted at 10,000  $\times g$  for 5 min. Total protein was quantitated using Non-Interfering Protein Assay (GenoTech, Tempe, AZ). Equal aliquots (200  $\mu\text{g}$ ) of total protein from cell lysates were immunoprecipitated with 1  $\mu\text{g}$  of antihuman COX-2 monoclonal antibody (Cayman Chemical, Ann Arbor, MI) and protein G. Protein was loaded into each lane on a 10% SDS-PAGE gel and separated. The gel was then

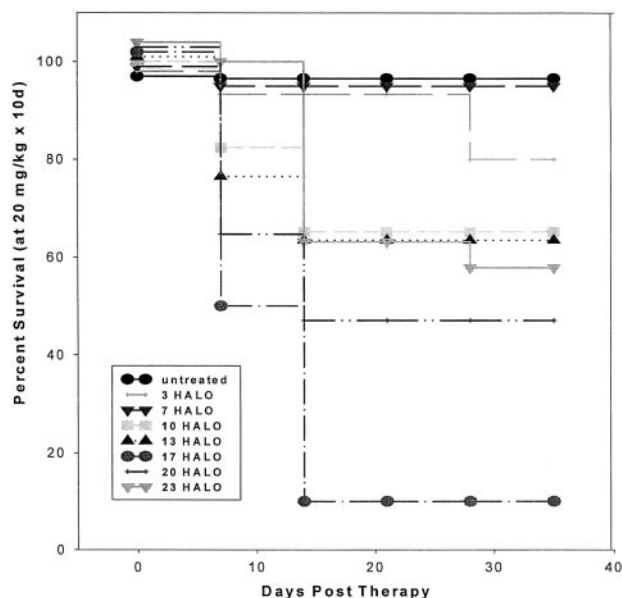


Fig. 1 Chronotolerance of tumor-bearing nude mice to a fixed i.p. dose (20 mg/kg) of celecoxib days 1-5 and 8-12 administered at either 3, 7, 10, 13, 17, 20, or 23 HALO. The percentage of survival of groups of 15-20 mice in each HALO group and  $n = 29$  in the untreated group is recorded as a function of time after the initiation of therapy.

electroblotted (24 V for 16 h) onto a polyvinylidene difluoride membranes (Schleicher and Schuell, Keene, NH). The membrane was blocked with 1% casein in PBS and then incubated at room temperature with an antihuman COX-2 polyclonal antibody (Cayman Chemical) at a 1:1000 dilution for 2 h. After washing, horseradish peroxidase-conjugated goat antimouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) was added at a 1:20,000 dilution for 1 h at room temperature. After washing, SuperSignal West DuraSubstrate (Pierce, Rockford, IL) was added to the membrane according to the manufacturer's instructions. Blots were exposed to film (Kodak MR) for 30 s to 5 min to reach the proper level of exposure.

## RESULTS

Chronotolerance and chronotherapy of celecoxib were investigated by administering a course of 20 mg/kg of the drug for 10 days (5 days followed by 2 days off and 5 days of dosing again). This schedule was used to give the animals a rest from the high doses of celecoxib being used and to reduce the likelihood of toxic side effects. Mice bearing matched-sized s.c. MCF-7, ZR75-30, or MDA-MB-468 breast xenografts were dosed at specific times of the day, either at 3, 7, 10, 13, 17, 20, or 23 HALO. Significant differences in survival were found, with the poorest survival noted at 17 HALO (5% on day 14) and at 20 HALO (48% on day 14). In contrast, the best survival was noted at 7 HALO (100% on day 35) and 3 HALO (82% on day 28; Fig. 1). The vehicle alone did not result in lethality at any time of day. Similar patterns of survival were observed with all three tumor lines. These results suggest that principles of chronotolerance clearly apply to celecoxib. Growth inhibition also varied between each HALO and between each tumor line.

Table 2 Percentage of growth inhibition in three breast xenografts on day 14 after therapy

	3 HALO	7 HALO	10 HALO	13 HALO	23 HALO
MCF-7	26.0	60.3	23.3	64.4	48.0
ZR75-30	46.4	8.3	8.3	23.8	57.1
MDA-MB-468	10.5	13.2	36.8	39.5	42.1

MCF-7 was most responsive to celecoxib at 7 HALO, with 65% growth inhibition on day 14 (Table 2). ZR-75-30 demonstrated less growth control than MCF-7 in response to this agent, with maximal effects occurring at 23 and 3 HALO (57 and 46% growth inhibition, respectively), and <10% growth inhibition observed at 7–10 HALO. In the MDA-MB-468 xenograft model, celecoxib was most effective at 23 HALO and at 10–13 HALO (42 and 36–39% GI, respectively) and showed almost no response at 3–7 HALO. Growth inhibition results are not presented for 17–20 HALO because of the extreme toxicity at these time points.

In an effort to explain the variability in survival as a function of time of dosing, animal body weight, peripheral WBCs, and serum renal and hepatic chemistry assays were performed. A 29% loss in body weight was observed with a 20-mg/kg dose of celecoxib given at 17 HALO. At all other times of dosing, mice experienced <13% body weight loss (Fig. 2, top;  $P < 0.001$  for 17 HALO compared with other HALO), suggesting that gastrointestinal toxicity may be the source of dose-limiting toxicity at 17 HALO only. Celecoxib was not particularly toxic to marrow, and minimal differences were seen as a function of dosing time. pWBCs were within 20% of untreated values (10,000 cells/mm<sup>3</sup>) at each HALO (Fig. 2, bottom).

Clinical chemistry assays from serum samples collected from mice on day 10 after the start of celecoxib therapy are shown in Fig. 3. No renal toxicity, as measured by uric acid (range, 1.0–2.0 mg/dl) or by BUN (range, 20.0–32.0 mg/dl), was noted. However, both hepatic markers showed an unusual significant decline in the 20 HALO group after therapy, which was not present in the 17 HALO group. Typically, a rise in chemical markers would indicate hepatic toxicity. In these studies, mean total bilirubin levels declined from 0.85 to 0.50 mg/dl ( $P < 0.02$ ), and mean AST declined from 280 to 115 mg/dl ( $P < 0.001$ ), suggesting that liver may, at least in part, be dose-limiting at 20 HALO.

Because a 20-mg/kg dose was lethal at some HALO, we performed a dose-finding study for each HALO by monitoring survival at three escalating doses per HALO at 5-mg/kg increments. The highest possible dose resulting in zero lethality and <20% body weight loss was called the MTD at that HALO. Fig. 4 summarizes the results and shows that the MTD varies between 10 mg/kg at 17 and 20 HALO and 25 mg/kg at 7 and 10 HALO. At 3, 10, and 13 HALO, the MTD was 20 mg/kg. On the basis of these corrected MTDs, we evaluated therapeutic effects in the MCF-7 tumor model, dosing mice with Celecoxib at the true MTD for each HALO. Fig. 5 illustrates the growth profiles of each treatment group for 6 weeks after the start of therapy. Maximal therapeutic response was found at 3, 7, and 23 HALO dosing with regressions seen at 28–35 days after therapy, al-

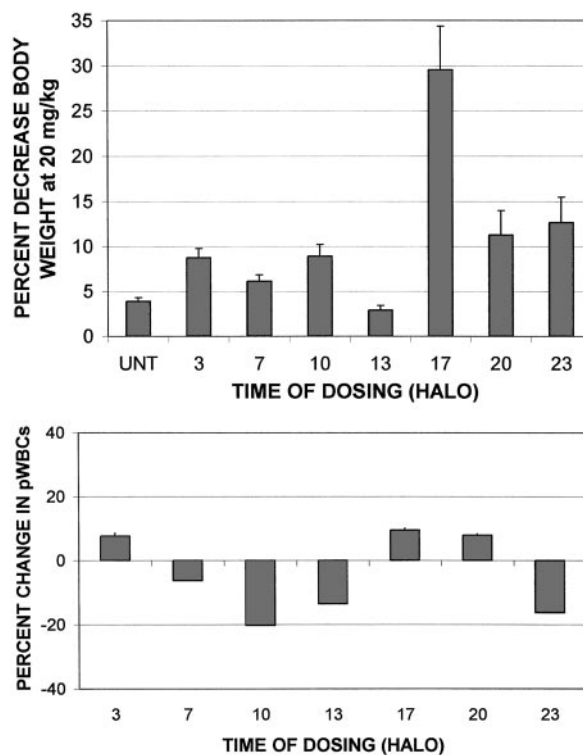


Fig. 2 Body weight change (upper panel) and peripheral WBCs (lower panel) 7 days after a regimen of celecoxib (20 mg/kg i.p. day 1–5) administered at 3, 7, 10, 13, 17, 20, or 23 HALO ( $n = 12$ /group). Bars, SD.

though no additional celecoxib was administered past day 12 ( $P = 0.037$  for HALO 3 versus untreated). Good growth control was also observed at 20 HALO, despite the lower MTD of 10 mg/kg. The worst response to celecoxib occurred at 17 HALO (10 mg/kg) and at 13 HALO (20 mg/kg), with tumor growth rate in treated mice exceeding the growth rate in untreated mice. Thus, therapeutic response does not appear to be dependent on dose of celecoxib and is strikingly different for each HALO.

We therefore questioned whether differences in growth in response to celecoxib dosing at each HALO was a function of the amount of COX-2 expressed in the tumor and whether toxicity at selected HALO could be explained by COX-2 expression in normal tissue. COX-2 expression at the gene and protein level was ascertained by RT-PCR and by Western blotting, and the results are presented in Fig. 6. MCA38 colon cancer cells were the positive control for both RT-PCR and Western blots. Sheep COX-2 was also a positive control for Western blots and cross-reacts with the antihuman COX-2 antibody. MCF-7 tumor COX-2 is expressed most strongly at the gene level at 20 HALO and to a lesser degree at 13 HALO. However, COX-2 protein was not found in MCF-7 tumors at any HALO, although celecoxib was shown to be therapeutic in these tumors at 20 through 7 HALO. COX-2 could not be found in cultured MCF-7 cells, although it was present in ZR75-30 and MDA-MB-468 breast cancer cells and in MCA-38 murine colon cancer cells. COX-2 was found in kidney and stomach at the gene level at various HALO but again could not be detected at

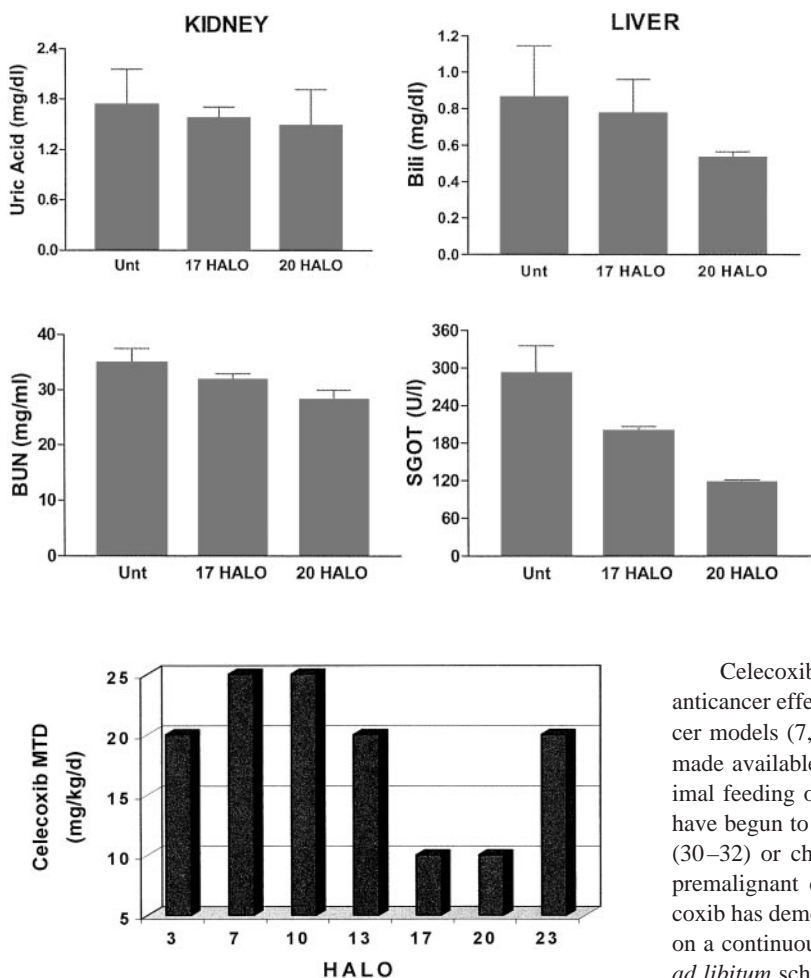


Fig. 3 Renal function (uric acid and BUN) and hepatic function (total bilirubin and AST) in untreated mice and 10 days after mice were treated at 17 or 20 HALO with celecoxib (20 mg/kg). Serum samples were analyzed using an ATAC6000 chemistry analyzer. Bars, SD.

Fig. 4 MTD determination of celecoxib (mg/kg) in athymic mice with MCF-7 s.c. tumors at each of the seven HALO of dosing. Three doses at 5-mg intervals were evaluated at each HALO. The MTD is the highest dose that results in no lethality and <20% weight loss.

the protein level. COX-2 was not expressed in liver or lungs at either the gene or the protein level (results not shown). These results suggest that the therapeutic effects and toxic side effects of celecoxib are occurring in a COX-2-independent manner and raise the question of what other target exists for celecoxib at the higher doses used here.

**DISCUSSION**

Circadian rhythms have been demonstrated both in animals and in humans in metabolically active normal tissues (gastrointestinal tract, bone marrow, gonads, epidermis, and cornea), as well as in most experimental tumors (solid and ascites) and human cancers. Finding times at which the therapeutic indices of standard, cytotoxic chemotherapeutic drugs are optimal has improved preclinical efficacy by up to three hundred % in tumor-bearing rodents (20). Randomized Phase III clinical trials have confirmed the overall ability to deliver higher doses of chemotherapy with concomitant improvements in clinical outcome by incorporating chronobiological principles (21, 22).

Celecoxib, a COX-2 inhibitor, has demonstrated moderate anticancer effects when administered alone in experimental cancer models (7, 13, 14, 29). In preclinical testing, it is typically made available in the feed throughout the day (13), with maximal feeding occurring in the night hours. Several laboratories have begun to study the efficacy of combined radiation therapy (30–32) or chemotherapy (33) with COX-2 inhibition. In the premalignant condition familial adenomatous polyposis, celecoxib has demonstrated efficacy at a dose of 400 mg twice a day on a continuous schedule for 6 months (34). Dosing was on an *ad libitum* schedule in this and other clinical studies performed with this agent. In an effort to further improve the utility of this agent alone or in combination with other modalities, we have examined the time dependence of the antitumor effects of celecoxib in three experimental breast tumor models to test the hypothesis that chronobiological modulation can further enhance the therapeutic effect in breast cancer. All mice were on the same 5 days on/2 days off/5 days on schedule of celecoxib. The only variable between treatment groups was the time of day when the dose was administered. We have identified times of the day when the MTD of celecoxib is 2.5-fold higher than at other times (25 mg/day at 7–10 HALO versus 10 mg/day at 17–20 HALO). Administering a 20 mg/kg dose at 17 HALO resulted in a ~29% body weight loss, whereas the same dose at any other time of day results in a <13% weight loss. The reason for these differences is not clear. Expression of COX-2 in normal tissues does not appear to be a factor, because COX-2 mRNA is expressed in stomach and kidney at select times but not in liver and lung, and COX-2 protein could not be found in any of these tissues at any time. These results suggest that the source of toxicity is some other tissue, and/or COX-2 may not be the target for celecoxib toxicity. Future pharmacokinetic studies will establish  $C_{max}$  and  $C_{min}$  at each dosing time. Absorption, distribution, clearance, and excretion of Celecoxib will then be established.

Most importantly, we have established optimal dosing



COX-2 in only select lines.<sup>4</sup> These results challenge the reported observations of other studies that have shown that the COX-2 inhibitor SC-58125 was effective at decreasing *in vitro* and *in vivo* cell growth only in a COX-2-expressing line (7). However, in a recent report, Williams *et al.* (35) have shown that the cytotoxic effects of high-dose celecoxib were independent of COX-2 inhibition, because similar effects were observed in COX-2 (+/+), COX-2 (+/-), and COX-2 (-/-) fibroblasts. Therefore, the therapeutic effects of high-dose celecoxib may be mediated by some mechanism other than COX-2.

Alternatively, low levels of COX-2, localized to microvascular endothelial cells and/or stromal fibroblasts (36, 37), may not be detectable on Western blots of whole tumor lysates but may mediate the celecoxib therapeutic effect. COX-2 has been shown to influence vascular endothelial growth factor expression, production of eicosanoid products (thromboxane A<sub>2</sub>, prostaglandin E<sub>2</sub>, and prostaglandin I<sub>2</sub>) that stimulate endothelial cell migration, and inhibit endothelial cell apoptosis by stimulation of bcl-2 or Akt activation (6, 13, 36–38). Differences in celecoxib efficacy in different tumor models may be a function of vascular development of the tumor and amount of microvascular COX-2.

Interestingly, the magnitude of growth inhibition and the optimal time of day to achieve good inhibition differ between the three breast tumor models studied. These differences may be a function of the amount of target (COX or some other, yet unidentified target) for celecoxib. Alternatively, the proliferation rate of the tumor and, therefore, the cell cycle status at any given HALO may be an important consideration. Differences in cell cycle-dependent expression of COX-2 (G<sub>1</sub>>S>G<sub>2</sub>-M) have been demonstrated recently (19). Another possibility is that dissimilarity in the vascular development and vascular physiology of the tumor (*e.g.*, microvessel density) that we have shown can be quite variable between tumor lines (39, 40) and is also influenced by circadian rhythms (26) and may provide an explanation for the observed unique results for each tumor.

There has been considerable debate concerning the utility of a mouse model toward understanding and applying chronobiological principles to patients. Although animal models have served an important function for assessing therapeutic potential and toxicity associated with a wide variety of treatments, application of results from nocturnal mice to humans may be complicated. Despite these difficulties, establishing principles based on circadian rhythms that could potentially improve therapeutic outcome is important. The ability to convert from mice to humans may eventually be possible by normalizing results with the periodicity of serum-immunomodulating hormones, such as cortisol or melatonin (41, 42, 43), or serum cytokines (44–46), because each has unique periodicities.

In summary, we have found that: (a) principles of chronotherapy and chronotoxicity identified for various chemotherapeutic agents apply to the COX-2 inhibitor celecoxib; (b) at 7–10 HALO, a dose of 25 mg/kg/day was tolerated, whereas at 17–20 HALO, the highest dose that could be given without lethality was 10 mg/kg/day; (c) a dose of 20 mg/kg/day resulted in 30% body weight loss when administered at 17 HALO but only 3–8% body weight loss at 3–13 HALO and only an 11–12% body weight loss at 20–23 HALO; (d) renal toxicity was not observed at any HALO at a 20-mg/kg/day dose of

celecoxib. Some hepatic toxicity may be present at 20 HALO at this dose; (e) the ability to give a higher dose of celecoxib at a particular HALO does not necessarily translate into a greater therapeutic effect (*e.g.*, 25 mg/kg/day at 10 HALO was less effective at controlling growth of MCF-7 tumors than 10 mg/kg/day at 20 HALO); and (f) toxicity could not be explained by COX-2 expression in normal tissues, nor could differences in therapeutic response at each HALO be explained by tumor COX-2 expression.

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# Clinical Cancer Research

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