Circadian and Melatonin Disruption by Exposure to Light at Night Drives Intrinsic Resistance to Tamoxifen Therapy in Breast Cancer

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Abstract

Resistance to endocrine therapy is a major impediment to successful treatment of breast cancer. Preclinical and clinical evidence links resistance to antiestrogen drugs in breast cancer cells with the overexpression and/or activation of various pro-oncogenic tyrosine kinases. Disruption of circadian rhythms by night shift work or disturbed sleep-wake cycles may lead to an increased risk of breast cancer and other diseases. Moreover, light exposure at night (LEN) suppresses the nocturnal production of melatonin that inhibits breast cancer growth. In this study, we used a rat model of estrogen receptor (ERα⁺) MCF-7 tumor xenografts to demonstrate how altering light/dark cycles with dim LEN (dLEN) speed the development of breast tumors, increasing their metabolism and growth and conferring an intrinsic resistance to tamoxifen therapy. These characteristics were not observed in animals in which the circadian melatonin rhythm was not disrupted, or in animals subjected to dLEN if they received nocturnal melatonin replacement. Strikingly, our results also showed that melatonin acted both as a tumor metabolic inhibitor and a circadian-regulated kinase inhibitor to reestablish the sensitivity of breast tumors to tamoxifen and tumor regression. Together, our findings show how dLEN-mediated disturbances in nocturnal melatonin production can render tumors insensitive to tamoxifen. Cancer Res; 74(15); 4099–110. ©2014 AACR.

Introduction

Approximately 60% to 75% of breast cancers express estrogen receptor (ER)-α and/or progesterone receptor (PR), which are markers and determinants for the use of endocrine therapies, including selective ERα modulators such as tamoxifen (TAM; refs. 1, 2), pure antiestrogens, and aromatase inhibitors (3–5). However, the development of resistance to tamoxifen and other endocrine therapies has become a major impediment in endocrine therapy such that anywhere from 30% to 50% of patients with ERα⁺ breast tumors display intrinsic resistance while most patients are initially responsive but will eventually develop acquired resistance to tamoxifen (6).

Compelling data have emerged from breast tumor biopsies and in vitro studies indicating that elevated expression and signaling of receptor tyrosine kinases, including members of the EGFR family and downstream MAPK/ERK and PI3K/AKT, can drive tamoxifen resistance through phosphorylation of either ERα at Ser167 or Ser118 to increase DNA binding or coregulator binding (7–12) to regulate cell proliferation and apoptosis. In addition, other signaling pathways are elevated or activated in tamoxifen-resistant breast tumors, including SRC, focal adhesion kinase (FAK), STAT3, and NF-κB (13, 14).

Numerous, studies have shown that the circadian melatonin signal regulates signaling and metabolic activities to inhibit breast cancer initiation, promotion, and progression (15–17). On the basis of early studies showing the anticancer actions of melatonin in breast cancer, Stevens (18) hypothesized that suppression of nighttime melatonin production by the pineal gland by light at night might explain the rise in breast cancer rates that have accompanied industrialization and electrification in the United States and other westernized countries. Light exposure at night (LEN) is a well-recognized environmental disruptor of the central circadian timing system located in the suprachiasmatic nucleus (SCN) of the brain (19). Nighttime production of melatonin by the pineal gland represents a highly reliable circadian output signal of the circadian clock whose suppression by LEN is intensity-, duration-, and wavelength dependent (20–22). These and other data led the World Health Organization to designate night shift work involving LEN-induced circadian/melatonin disruption as a probable carcinogen (class 2a) and risk factor for the development of breast cancer (23). Furthermore, using our novel tissue-isolated MCF-7 human...
breast cancer xenograft model in circadian/melatonin intact female nude rats, we also report increases in tumor growth rates and enhanced ERK, AKT, and AKT stimulatory 3-phosphoinositide-dependent kinase-1 activity (24–27), and repression of GSK3β activity in breast tumor xenografts in response to light exposure during the night or day and that these changes are blocked by melatonin (28).

The above studies, in addition to those demonstrating that physiologic nocturnal concentrations of melatonin significantly increase the sensitivity of ERαþ MCF-7 human breast cancer cells to tamoxifen in vitro (29), prompted us to postulate that dim light exposure at night (dLEN), by virtue of its ability to suppress nocturnal melatonin production, will promote partial or complete resistance to tamoxifen therapy in vivo. In the present study, we demonstrate for the first time that exposure to dLEN, via suppression of the nighttime melatonin signal, induces tumor progression and intrinsic resistance to tamoxifen in human breast tumor xenografts. Furthermore, the maintenance of the endogenous melatonin signal or its replacement with exogenous melatonin under dLEN conditions preserves tamoxifen sensitivity and drives tumor regression.

Materials and Methods

Chemicals and reagents

All chemicals and tissue culture reagents were purchased from Sigma-Aldrich. Cell culture medium, RPMI-1640, and FBS were purchased from Invitrogen Corporation. High-performance liquid chromatography (HPLC)-grade reagents were purchased from Fisher Chemical. Free fatty acid, cholesterol ester, triglyceride, phospholipid, rapeseed oil methyl ester, fluoride-methanol, potassium chloride, sodium chloride, perchloric, and trichloroacetic acids were purchased from Sigma-Aldrich. The HPLC standards, (±)-5-HETE, and (±)-13(S)-HODE were purchased from Cayman Chemical Co.

Cell line and cell culture

The ERαþ /PRþ, tamoxifen-sensitive MCF-7 human breast cancer cell line (passage numbers 18–20) used in these studies was obtained from American Tissue Culture Collection: ATCC. These cells were tested and authenticated by ATCC and immediately expanded, and frozen down as stock for future studies. Cells from low passage frozen stocks (passage numbers 18–20) were used in these studies. Briefly, cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 50 mmol/L minimum essential medium nonessential amino acids, 1 mmol/L sodium pyruvate, 2 mmol/L glutamine, 10 mmol/L basal medium eagle, 100 mg/mL streptomycin, and 100 U/mL penicillin and maintained at 37°C in a humidified atmosphere of 5% CO2 and 95% air.

Animals, housing conditions, and diet

Female athymic, inbred nude rats (Hsd:RH–Foxn1rnu), 1 to 2 weeks of age, used in this study were purchased from Harlan Laboratories. Upon arrival, animals were maintained in environmentally controlled rooms (25°C; 50%–55% humidity) with controlled diurnal lighting schedule of 12-hour light:12-hour dark at subjective night (LD 12:12; 300 lux; 125 μW/cm²; lights on, 06:00 hours, and off at 18:00 hours). Animal rooms were completely devoid of light contamination during the dark phase (30). One week before tumor implantation, two-thirds of the animals were switched to a 12-hour light:12-hour dim light at night, subjective night (dLEN) cycle (0.2 lux, with lights on at 06:00 hours and off at 18:00 hours, and dLEN on at 18:00 hours and off at 06:00 hours) in an Assessment and Accreditation of Laboratory Animal Care International-accredited facility and in accordance with The Guide. Animals were given free access to food (Purina 5053 Irradiated Laboratory Rodent Diet) and acidified water as previously described (30, 31). All procedures used for animal studies were approved by the Tulane University Institutional Animal Care and Use Committee (New Orleans, LA).

Arterial blood collection

Diurnal plasma melatonin levels (pg/mL; mean ± 1 SD) of naïve, female nude rats (n = 12) maintained initially in the control LD 12:12 cycle (300 lux; 125 μW/cm²; lights on, 06:00 hours) or in the dLEN cycle were measured as previously described (30, 31). In experimental animals during the course of these studies, blood was collected over time at six circadian time points (04:00, 08:00, 12:00, 16:00, 20:00, and 24:00 hours) for the measurement of levels of melatonin, total fatty acids, glucose, and lactic acid (26, 32).

MCF-7 tumor xenografts development in nude mice

Ovariectomized athymic nude mice (4–5-week-old females) were obtained from Charles River and maintained in pathogen-free aseptic conditions with phytoestrogen-free food and water ad libitum. All mice were supplemented with estrogen pellets (0.72 mg of 17β-estradiol 60-day release from Innovative Research of America) and estradiol-dependent MCF-7 xenografts were propagated. Exponentially growing MCF-7 cells (Passage numbers 18–20) were harvested and approximately 5 × 10⁶ MCF-7 cells in 150 μL of PBS-Matrigel mixture were orthotopically and bilaterally implanted into the mammary fat pads of female nude mice, as previously described (32).

Tumor transplantation into athymic nude rats

After one week of photoperiod acclimation (LD 12:12 or dLEN for study I: dLEN or dLEN þ nighttime melatonin supplementation for study II), nude rats were implanted in a tissue-isolated fashion with (ERαþ) MCF-7 human tumor xenografts obtained from the tumor xenografts initially developed in mice, as described previously (24, 26, 31). Once implanted tissue-isolated tumor xenografts reached a palpable size (approximately pea size) in the nude rats, tumors were measured every day for estimated tumor weights, as described previously (24, 27, 31).

Tumor growth studies

Two separate approaches to evaluate the effects of dLEN and melatonin on the responsiveness of breast tumor xenografts to 4OH-TAM were used as shown in Fig. 1. In study I, tissue-isolated (ERαþ) MCF-7 human breast tumor xenografts were implanted into nude rats maintained in a control LD 12:12 (elevated endogenous nocturnal melatonin) lighting schedule...
These lighting environments (injection. The second group of animals was maintained in either the dLEN lighting or dLEN lighting schedules. 3, administration of vehicle or 4OH-TAM was initiated as tumors in each lighting schedule reached an estimated weight of 2.5 g. 4, as tumors reached 8 g (estimated weight), regressed to their nighttime drinking water (0.1 g/kg/d) such that they received approximately 2.5 μg melatonin daily intake, based on their nighttime drinking water (0.1 g/mL) via intraperitoneal injection. Thus this study consisted of four groups: group I (dLEN treated with vehicle); group II (dLEN treated with tamoxifen); group III (dLEN supplemented with melatonin and treated with vehicle); and group IV (dLEN supplemented with melatonin and treated with tamoxifen).

A-V tumor measurements
When tumors reached an estimated weight of 8 g, (groups I, II, and III), were out 40 days postimplantation, or regressed to 1.6 g (group IV) following tamoxifen-administration, tumors were prepared for in situ tumor vein cannulation (31). Experiments were conducted between 24:00 and 04:00 hours following a normal nocturnal feeding period. Animal preparation, including anesthesia administration and blood sample collection, was described previously (24, 27, 31). Analysis of arterial glucose, lactate, acid/gas, fatty acids, and melatonin was conducted as previously described (30).

Tumor lysate extraction and Western blot analysis
Frozen tumors were pulverized and homogenized in RIPA buffer (1 × PBS, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS) containing a protease inhibitor cocktail (Sigma Scientific) and phosphatase inhibitor cocktails. Total tumor protein was isolated from tumor lysates as previously described (28) and aliquots stored at −80°C. One hundred and twenty micrograms of protein from each sample were separated on a criterion precast gel (Bio-Rad) and transferred onto nitrocellulose membranes (Bio-Rad). After incubation with 5% nonfat milk in TBS containing 0.1% Tween 20, immunoblot analyses were probed with various antibodies, including cleaved caspase-3, phospho-(p)-ERK1/2 Thr202/Tyr204, total (t)-ERK1/2, p-AKT Ser473, t-STAT3 Tyr707 and p-STAT3 Tyr707, t-CREB, p-FAK Tyr576/577, t-FAK, p-CREB Ser133, SRC Tyr416, t-SRC, p-NF-kB, p-Bcl2, and t-Bcl2, with antibody antibodies to α-tubulin and α-actin (Sigma) to evaluate loading. Quantitation of Western blot analyses and differences in expression of total and phosphorylated proteins were determined by digital quantitation of phosphorylated and total protein levels and normalizing phosphorylated levels to the levels of the total protein of interest and comparing the levels of tamoxifen treated, LD 12:12, dLEN-exposed group versus the control group at circadian time points assessed by the Student t test. Statistical differences among group means in tumor perfusion studies were determined by digital quantitation of phosphorylated and total protein levels and normalizing phosphorylated levels to the levels of the total protein of interest and comparing the levels of tamoxifen treated, LD 12:12, dLEN-exposed group versus the control group at circadian time points assessed by the Student t test. Statistical differences among group means in tumor perfusion studies were evaluated by Student t test. Statistical differences among group means in tumor perfusion studies were evaluated by Student t test. Statistical differences among group means in tumor perfusion studies were evaluated by Student t test.

Study I
When tumor weights reached approximately 2.5 g estimated weight, one half of the animals (n = 3/group) maintained in either the dLEN lighting or LD 12:12 lighting environments were treated daily at 16:00 hours with either 0.1 mL of 4OH-TAM (80 μg/kg/d) or diluent by intraperitoneal injection. Thus this study consisted of four groups: group I (dLEN treated with vehicle); group II (dLEN treated with tamoxifen); group III (dLEN supplemented with melatonin and treated with vehicle); and group IV (dLEN supplemented with melatonin and treated with tamoxifen).

Study II All animals were maintained in the dLEN lighting schedule and were randomized so that half received a vehicle at nighttime and the other half received a supplement of melatonin in their nighttime drinking water (0.1 μg/mL) such that they received approximately 2.5 μg melatonin daily intake, based on a daily water intake of about 25 mL, which simulates the high normal nighttime physiologic levels of melatonin. When tumor weights reached approximately 2.5 g estimated weight, one half of the animals (n = 3/group) maintained in either the dLEN lighting or dLEN supplemented with melatonin were treated daily at 16:00 hours with either 0.1 mL of 4OH-TAM (80 μg/kg/d) or diluent by intraperitoneal injection. Thus this study consisted of four groups: group I (dLEN treated with vehicle); group II (dLEN treated with tamoxifen); group III (dLEN supplemented with melatonin and treated with vehicle); and group IV (dLEN supplemented with melatonin and treated with tamoxifen).
determined by a one-way ANOVA followed by Bonferroni multiple comparison test. Differences in the tumor growth rates among groups were determined by regression analyses and tests for parallelism (Student t test). Differences were considered to be statistically significant at $P < 0.05$.

Results

Study I

Plasma melatonin levels. Figure 2A shows that under LD 12:12 conditions, before tamoxifen treatment, plasma melatonin levels increased and remained high during the dark phase reaching a peak at 24:00 hours that was more than 70-fold higher than during the light phase. In rats under dLEN lighting schedule, melatonin levels remained consistently low to undetectable throughout the 24-hour period (Fig. 2B).

*dLEN* promotes tumor growth and intrinsic resistance to 4OH-TAM in (ERa+) MCF-7 tissue-isolated breast tumor xenografts. Figure 3A shows that breast tumor xenografts from rats housed in dLEN had a significantly reduced ($P < 0.001$) latency-to-onset and a significantly faster ($P < 0.001$) growth rate (2.6-fold, 0.73 g/d) compared with tumor xenografts grown under the LD 12:12 lighting schedule (0.28 g/d).

Tumors from rats in dLEN showed complete intrinsic resistance to tamoxifen growing at the same rate (0.69 g/d) as vehicle-treated xenografts in dLEN. However, late afternoon (16:00 hours/4:00 pm) administration of tamoxifen significantly inhibited ($P < 0.001$) tumor growth in rats housed in LD 12:12 as tumors regressed at a rate of $-0.14$ g/d. Figure 3A also shows visually the dynamic difference in tumor growth and regression between xenografts from dLEN with or without melatonin supplementation and LD 12:12 groups treated with tamoxifen.

Tumor cAMP levels. Tumor cAMP levels were 14- and 20-fold higher at the mid-dark phase in rats in vehicle- and tamoxifen-treated dLEN rats, respectively, as compared with the same treatment groups on LD 12:12 (Table 1). Administration of 4OH-TAM to rats in dLEN did not affect tumor cAMP levels; however, in LD 12:12, it further diminished tumor cAMP levels by 40% as compared with vehicle-treated animals.

Tumor linoleic acid uptake, 13-HODE production, and proliferative activity. Tumor linoleic acid (LA) uptake and 13-HODE formation were completely suppressed during the mid-dark phase in both vehicle- and tamoxifen-treated dLEN rats on LD 12:12, while high levels of tumor LA metabolism were seen in both vehicle- and tamoxifen-treated rats maintained on dLEN (Table 1). In addition, tumor incorporation of [3H]-thymidine into DNA in vehicle- and tamoxifen-treated dLEN rats was elevated by 10-fold at the mid-dark phase as compared with vehicle- and tamoxifen-treated rats on LD 12:12, respectively. No difference was observed between vehicle- and tamoxifen-treated groups in both photoperiods.

Warburg effect - tumor glucose and O2 uptakes, and lactic acid and CO2 production. Tumor glucose and O2 uptake were increased by 2- and 1.5-fold, respectively, at 24:00 hours (mid-dark phase) in vehicle-treated dLEN rats as compared with vehicle-treated rats on the standard LD 12:12 photo-
in vehicle-treated and 3.5- and 2.7-fold, respectively, in tamoxifen-treated LD dLEN rats as compared with vehicle- and tamoxifen-treated rats in LD 12:12, respectively (Table 2). Tumor lactate and CO₂ production were reduced by 29% in LD 12:12 rats, and by 93% in tamoxifen-treated rats on LD 12:12.

Modulation of apoptosis in breast tumor xenografts exposed to dLEN or nighttime melatonin and treated with 4OH-TAM. Caspase-3 has high homology to the CED-3 protease and is a key player in apoptosis. Caspase-3 is cleaved by caspases-8 and -9 into two bands of (17 and 19 kDa) that in turn induce apoptosis. The increased levels of cleaved caspase-3 are...
Table 1. Tumor cAMP levels, LA uptake, 13-HODE formation, [3H]thymidine incorporation into DNA, and DNA content during the mid-dark phase (24:00 hours) in tissue-isolated MCF-7 (ERα+) human breast cancer xenografts in nude female rats (study I) exposed to either LD 12:12 or dLEN and treated with either vehicle or tamoxifen (80 mg/kg/d).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>cAMP (nmol/g tissue)</th>
<th>13-HODE formation (ng/min/g)</th>
<th>DNA content (mg/g DNA)</th>
<th>LA uptake (g/min/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dLEN vehicle</td>
<td>1.55 ± 0.28</td>
<td>3.89 ± 0.34</td>
<td>0.11 ± 0.02</td>
<td>0.11 ± 0.02</td>
</tr>
<tr>
<td>dLEN tamoxifen</td>
<td>1.38 ± 0.14</td>
<td>3.94 ± 0.49</td>
<td>0.11 ± 0.05</td>
<td>0.11 ± 0.05</td>
</tr>
<tr>
<td>LD 12:12 vehicle</td>
<td>0.11 ± 0.02</td>
<td>0.11 ± 0.02</td>
<td>0.11 ± 0.02</td>
<td>0.11 ± 0.02</td>
</tr>
<tr>
<td>LD 12:12 tamoxifen</td>
<td>0.11 ± 0.02</td>
<td>0.11 ± 0.02</td>
<td>0.11 ± 0.02</td>
<td>0.11 ± 0.02</td>
</tr>
</tbody>
</table>

NOTE: Values are mean ± SD (n=3/group).

**Study I**

**Modulation of key proliferation and survival signaling molecules in breast tumor xenografts exposed to dLEN or nighttime melatonin with 4OH-TAM.** Tumors from animals in dLEN treated with vehicle or tamoxifen, harvested at 24:00 hours (mid-dark phase, subjective night) showed high levels of ERK1/2 phospho-activation, whereas tumors from animals in LD 12:12 with elevated nighttime melatonin showed almost complete suppression of ERK1/2 phospho-activation (Fig. 5A). No change in AKT, either total or phosphorylated at ser473 (p-AKT<sup>473</sup>) or NF-kβ/p65, was observed in response to dLEN or LD 12:12 in either the presence or absence of tamoxifen.

Tumors from dLEN groups (vehicle or tamoxifen treated) also showed elevated levels of p-SRC and p-FAK at sites Y416 and Y576/577, respectively, but repression of the phospho-active forms was seen in LD 12:12 tumors (Fig. 5A). The levels of p-STAT3 (Y705) were also elevated in dLEN tumors treated with vehicle or tamoxifen, but were almost completely inhibited in LD 12:12 tumors (vehicle and tamoxifen treated). Figure 5A shows cAMP—responsive element binding protein (CREB), a transcription factor phospho-activated by cAMP/PKA and upregulated in many breast tumors (33–35), is phospho-activated in dLEN tumors treated with or without tamoxifen, but inhibited in LD 12:12 tumors.

**dLEN activation and melatonin repression of ERα phosphorylation and activation.** Elevated phosphorylation of Ser118 and Ser167 of the ERα was observed in tumors from the dLEN group without significant change in total ERα protein levels (Fig. 5A) compared with those in the LD 12:12 groups.

**Study II**

**Plasma melatonin levels in study II.** Melatonin levels remained low throughout the 24-hour period in rats under the dLEN lighting schedule before melatonin and/or tamoxifen treatment. As shown in Fig. 2B, following administration of melatonin in the drinking water (2.5 mg/d) plasma melatonin levels at 24:00 hours (mid-dark phase/subjective night) were 70- to 90-fold more than at 12:00 hours in control (dLEN) rats, showing visually the dramatic response (P < 0.0001) to 4OH-TAM administered at 1600 hours (4:00 pm), regressing at a rate of ~0.17 g/d. Figure 3B shows the visual dramatic markers of apoptosis in study I (Fig. 4A) and showed a significant 28% and 42% increase in LD 12:12 tumors with or without 4OH-TAM treatment, respectively, versus dLEN tumors.

**dLEN promotes tumor growth and intrinsic resistance to 4OH-TAM in (ER<sup>+</sup>) MCF-7 tissue-isolated breast tumor xenografts.** Figure 3B demonstrates that (ER<sup>+</sup>) MCF-7 breast tumor xenografts from rats housed in dLEN had a significantly shorter (P < 0.001) latency-to-onset and a significantly increased growth rate (2.2-fold) compared with tumors in rats in dLEN that were supplemented with exogenous melatonin at night. Tumor xenografts from rats in dLEN showed complete intrinsic resistance to 4OH-TAM, growing at the same rate as vehicle-treated dLEN xenografts. Tumor xenografts from rats in dLEN supplemented with nighttime melatonin shows visually the dramatic response (P < 0.0001) to 4OH-TAM administered at 1600 hours (4:00 pm), regressing at a rate of ~0.17 g/d. Figure 3B shows the visual dramatic
difference in tumor growth and regression between the various dLEN groups.

**Tumor cAMP levels.** Table 3 shows that tumor cAMP concentrations at 24:00 hours in dLEN animals given either vehicle or tamoxifen were 10- and 27-fold higher, respectively, than in the same treatment groups in dLEN rats supplemented with nighttime melatonin. In rats in dLEN supplemented with melatonin, tamoxifen administration further decreased tumor cAMP levels by 63% compared with vehicle controls.

**Tumor linoleic acid uptake, 13-HODE production, and proliferative activity.** Table 3 shows elevated levels of tumor LA uptake and 13-HODE formation at 24:00 hours (mid-dark phase) in both dLEN groups (vehicle and tamoxifen treated) without melatonin supplementation. However, uptake of LA and its conversion to 13-HODE were completely suppressed at 24:00 hours in dLEN groups supplemented with nighttime melatonin. Furthermore, incorporation of [3H]-thymidine into the DNA of dLEN tumors, both vehicle- and 4OH-TAM-treated, was elevated by 10-fold compared with the same treatment groups in dLEN tumors supplemented with melatonin.

**Warburg effect: tumor glucose and O2 uptakes, and lactic acid and CO2 production.** In Table 4, tumor glucose and O2 uptake increased by 2.8- and 1.3-fold and 2.3- and 3.4-fold, in vehicle- and tamoxifen-treated dLEN rats, respectively, as compared with the same treatment groups from dLEN melatonin-supplemented tumors. Also, tumor lactate and CO2 production increased by 2.7- and 1.8-fold and 1.3- and 2.9-fold in vehicle- and tamoxifen-treated dLEN rats, respectively, versus vehicle- and tamoxifen-treated dLEN melatonin-supplemented rats.

**Modulation of apoptosis and autophagy in breast tumor xenografts exposed to dLEN or nighttime melatonin and treated with 4OH-TAM.** Caspase-3 cleavage, a marker of apoptosis, was increased by 15% and 23% in dLEN melatonin-supplemented tumors treated with vehicle or 4OH-TAM, respectively, compared with the same treatment groups in dLEN tumors without melatonin supplementation (Fig. 4B). Furthermore, an increase in the conversion of LC3B-I (upper band) to LC3BII (lower band) is observed in dLEN melatonin-supplemented tumors treated with vehicle or 4OH-TAM versus xenografts compared with dLEN tumors treated with vehicle or 4OH-TAM (Fig. 4C).

**Modulation of key proliferation and survival signaling molecules in breast tumor xenografts exposed to dLEN or nighttime melatonin treated with 4OH-TAM.** In study II, dLEN tumors treated with diluent or 4OH-TAM showed high levels of phospho-active ERK1/2, SRC, FAK, STAT3, and CREB (Fig. 5B) and modest, but consistent, induction of phospho-active AKT[Ser473]. A large and consistent increase in t-NF-kB/p65 was also observed in dLEN tumors treated with vehicle or 4OH-TAM. Conversely, the corresponding treatment groups in dLEN melatonin-supplemented tumors showed almost complete suppression of p-ERK1/2, p-AKT, p-SRC, p-FAK, p-STAT3, and p-CREB, and a significant reduction of t-NF-kB/p65 was also observed.
dLEN activation and melatonin repression of ERα phosphorylation and activation. Elevated phosphorylation of Ser118 and 167 was observed in tumors from rats house in dLEN, without significant change in total ERα protein levels (Fig. 5C). Supplementation with exogenous nighttime melatonin in dLEN tumors greatly suppressed ERα phosphorylation at Ser118 and 167 by 68% and 76%, respectively.

Discussion

Unlike exposure to bright light at night, which both disrupts the activity of the central circadian clock and suppresses pineal melatonin synthesis, dLEN suppresses only melatonin production while normal SCN-driven circadian feeding and drinking activity persist (24). In these studies, dLEN was at a light intensity of 0.2 lux, which is equivalent to a crack of light under a door in a completely dark room. As shown in study I, female nude rats on LD 12:12 lighting schedule evinced a circadian rhythm of plasma melatonin, that closely mimics the normal melatonin circadian profile in adult human female subjects (26, 31), whereas dLEN only suppressed the nocturnal melatonin signal as we previously reported (24, 26). Study II demonstrated that supplementation of the drinking water with
melatonin is an effective replacement strategy for reconstituting nighttime levels of melatonin in dLEN rats. This study clearly demonstrates that under dLEN conditions, latency-to-tumor onset was shortened while the growth of tumors and their development of intrinsic tamoxifen resistance were stimulated. Conversely, in the presence of the nocturnal circadian melatonin signal, in response to dLEN-induced circadian/melatonin disruption, is an underlying and novel mechanism for the development of intrinsic resistance to tamoxifen therapy. We anticipated that the tumor regression driven by the combination of melatonin and 4OH-TAM would be associated with significant increases in apoptosis and autophagy. However, only moderate increases in apoptosis and autophagy were observed at the mid-dark phase time point, which do not interfere with the growth of tumors and their development of intrinsic tamoxifen resistance but also confers increased tumor sensitivity to tamoxifen. These findings are supported by our earlier in vitro work (29) and demonstrate that the loss of the nocturnal circadian melatonin signal, in response to dLEN-induced circadian/melatonin disruption, is an underlying and novel mechanism for the development of intrinsic resistance to tamoxifen therapy.

Table 2. Tumor uptake of glucose and O2 and production of lactate and CO2 production in vivo (Warburg effect) during the mid-dark phase (24:00 hours) in tissue-isolated MCF-7 (ERα+) human breast cancer xenografts in nude female rats (study I) exposed to either LD 12:12 or dLEN and treated with either vehicle or tamoxifen (80 μg/kg/d).

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Glucose uptake (μg/min/g)</th>
<th>Lactate release (nmol/min/g)</th>
<th>O2 uptake (% of supply)</th>
<th>CO2 production (% of original value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dLEN vehicle</td>
<td>4.3 ± 0.1 (32.8 ± 5.7%)b</td>
<td>28.9 ± 4.4</td>
<td>75.8 ± 1.1</td>
<td>136.2 ± 10.5</td>
</tr>
<tr>
<td>dLEN tamoxifen</td>
<td>5.1 ± 1.0 (31.4 ± 3.5%)b</td>
<td>30.2 ± 5.1</td>
<td>76.5 ± 0.9</td>
<td>133.0 ± 4.3</td>
</tr>
<tr>
<td>LD 12:12 vehicle</td>
<td>2.3 ± 0.2c (12.9 ± 1.5%)bc</td>
<td>15.9 ± 0.8c</td>
<td>50.5 ± 3.5c</td>
<td>69.4 ± 3.1c</td>
</tr>
<tr>
<td>LD 12:12 tamoxifen</td>
<td>1.3 ± 0.4d (2.8 ± 1.5%)bcd</td>
<td>2.3 ± 0.3d</td>
<td>21.9 ± 3.3d</td>
<td>49.2 ± 2.4d</td>
</tr>
</tbody>
</table>

NOTE: Values are mean ± SD (n = 3/group).
*Three animals (tumors)/group; (±SD) tumor weights, MCF-7 (ERα+) dLEN group, vehicle treatment = 7.26 ± 0.21 g; tamoxifen treatment = 7.08 ± 0.22 g; LD 12:12 group vehicle treatment = 5.53 ± 0.20 g; tamoxifen treatment = 1.95 ± 0.09 g, respectively. All tumors were harvested during the mid-dark phase at 24:00 hours.

Table 3. Tumor cAMP levels, LA uptake, 13-HODE formation, [3H]thymidine incorporation into DNA, and DNA content during the mid-dark phase (24:00 hours) in tissue-isolated MCF-7 (ERα+) human breast cancer xenografts in nude female rats (study II) exposed to dLEN and treated with either vehicle, tamoxifen (80 μg/kg/d), melatonin (2.5 μg/d), or tamoxifen + melatonin.

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>cAMP (nmol/g tissue)</th>
<th>LA uptake (μg/min/g)</th>
<th>13-HODE (ng/min/g) Arterial supply</th>
<th>13-HODE (ng/min/g) Venous output</th>
<th>[3H]-Thymidine incorporation (dpm/ng DNA)</th>
<th>DNA content (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dLEN vehicle</td>
<td>1.63 ± 0.20</td>
<td>2.5 ± 0.7 (35.3 ± 1.5%)b</td>
<td>0</td>
<td>6.9 ± 0.7</td>
<td>67.4 ± 1.1</td>
<td>3.5 ± 0.2</td>
</tr>
<tr>
<td>dLEN tamoxifen</td>
<td>1.62 ± 0.22</td>
<td>2.6 ± 0.5 (34.4 ± 2.8%)b</td>
<td>0</td>
<td>6.8 ± 0.5</td>
<td>69.2 ± 2.3</td>
<td>3.4 ± 0.2</td>
</tr>
<tr>
<td>dLEN melatonin</td>
<td>0.16 ± 0.06c</td>
<td>0</td>
<td>0</td>
<td>5.8 ± 1.0c</td>
<td>2.0 ± 0.1c</td>
<td>2.0 ± 0.1c</td>
</tr>
<tr>
<td>dLEN tamoxifen + melatonin</td>
<td>0.06 ± 0.02d</td>
<td>0</td>
<td>0</td>
<td>5.8 ± 0.1d</td>
<td>2.0 ± 0.1d</td>
<td>2.0 ± 0.1d</td>
</tr>
</tbody>
</table>

NOTE: Values are mean ± SD (n = 3/group).
*Three animals (tumors)/group; (±SD) tumor weights, in MCF-7 (ERα+) dLEN groups, vehicle treatment = 7.26 ± 0.21 g; tamoxifen treatment = 7.08 ± 0.22 g; dLEN melatonin treatment = 5.53 ± 0.20 g; dLEN tamoxifen + melatonin treatment = 1.95 ± 0.09 g, respectively. All tumors were harvested during the mid-dark phase at 24:00 hours.

*expressed in parenthesis as % of arterial LA supply.
*P < 0.05 versus vehicle.
*P < 0.05 versus tamoxifen and melatonin.
*P < 0.05 versus tamoxifen.
amplified in the present studies, it is possible that dLEN may have of these same pathways were phospho-activated in response to NF-

Numerous studies have linked the activation of ERK/12, AKT, activity during the day in human breast cancer xenografts. characterizes by suppressed activity during the night and peak
cell proliferation/survival, are important in supplying both the Warburg effect as well as the uptake of LA and its 
presses the metabolism of glucose via the inhibition of aerobic 

breast cancer growth progression and tamoxifen resistance. 

Although the evolving list of mechanisms by which melatonin inhibits human breast cancer growth includes the inhibition of multiple signaling, transcriptional and metabolic pathways, the mechanism(s) by which this indoleamine increases breast cancer responsiveness to tamoxifen is unknown. Elucidating this mechanism(s) is essential for understanding how dLEN-induced melatonin suppression promotes breast cancer growth progression and tamoxifen resistance. 

With respect to breast cancer metabolism, melatonin suppresses the metabolism of glucose via the inhibition of aerobic glycolysis (Warburg effect) as well as the uptake of LA and its metabolism to its mitogenic end product 13-HODE. Both of these processes, which are linked to key signaling pathways for cell proliferation/survival, are important in supplying both the energetics and infrastructure involved in building tumor biomass (35). We recently reported (36) that in human breast cancer xenografts LA metabolism, the Warburg effect, their associated cell signaling pathways, and cell proliferative activity exhibit robust melatonin-driven circadian rhythms characterized by suppressed activity during the night and peak activity during the day in human breast cancer xenografts. Numerous studies have linked the activation of ERK/12, AKT, NF-kB, cAMP SRC, STAT-3, and I Activate the potentiation of the pERK1/2, p-SRC, p-FAK, and p-STAT3 pathways is supported by the work of Hiscox and colleagues (43) showing that inhibition of c-SRC suppresses the expression of cyclin D1 and c-Myc, and thus inhibits the emergence of a tamoxifen-resistant phenotype in breast cancer cell lines. Future studies employing multiple circadian time points will be required to determine the precise temporal sequence of kinase and transcription factor phosphorylation in defining the efficacy of endocrine therapies, depending on the timing of their administration. 

It is important to note that STAT3 is regarded as a point of convergence for many oncogenic signals and its aberrant constitutive activity is crucial for the survival, proliferation, and metastatic activity of tumors of different origins (44). Moreover, STAT3 activity can be driven by activation of phospho-active ERK1/2, cAMP, SRC, and IL6. On the basis of the fact that the Warburg effect can drive some tumors to chemoresistance (40), it is conceivable that dLEN may drive breast cancer to innate resistance to tamoxifen via constitutive activation of the Warburg effect.

On the other hand, in animals exposed to LD 12:12, with an intact endogenous melatonin signal, or to dLEN and receiving melatonin-replacement therapy, it is also possible that the rapid tumor regression observed in response to 4OH-TAM therapy can be primarily ascribed to inhibition of key proliferative and survival signaling pathways. Our results clearly demonstrate that the nocturnal exposure to melatonin dramatically suppresses or ablates the expression of total or phospho-activated ERK1/2, AKT, cAMP, SRC, FAK, STAT3, CREB, and NF-kB. Numerous reports have described the importance of each of these pathways, either individually or collectively, in driving breast cancer toward proliferation, progression, and tamoxifen resistance (41, 42). The potential importance of melatonin as a natural endogenous nighttime inhibitor of the pERK1/2, p-SRC, p-FAK, and p-STAT3 pathways is supported by the work of Hiscox and colleagues (43) showing that inhibition of c-SRC suppresses the expression of cyclin D1 and c-Myc, and thus inhibits the emergence of a tamoxifen-resistant phenotype in breast cancer cell lines. Future studies employing multiple circadian time points will be required to determine the precise temporal sequence of kinase and transcription factor phosphorylation in defining the efficacy of endocrine therapies, depending on the timing of their administration. 

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Table 4. Tumor uptake of glucose and O2 and production of lactate and CO2 production in vivo (Warburg effect) during the mid-dark phase (24:00 hours) in tissue-isolated MCF-7 (ERα+) human breast cancer xenografts in nude female rats (study II) exposed to dLEN and treated with either vehicle, tamoxifen (80 μg/kg/d), melatonin (2.5 μg/d), or tamoxifen + melatonin

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Glucose uptake (µmol/min/g)</th>
<th>Lactate release (nmol/min/g)</th>
<th>O2 uptake (% of supply)</th>
<th>CO2 production (% of original value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dLEN vehicle</td>
<td>4.4 ± 0.5 (33.7 ± 2.8%)ab</td>
<td>27.3 ± 1.1</td>
<td>78.9 ± 0.8</td>
<td>133.6 ± 8.7</td>
</tr>
<tr>
<td>dLEN tamoxifen</td>
<td>4.4 ± 0.5 (34.5 ± 1.7%)ab</td>
<td>25.8 ± 2.4</td>
<td>78.6 ± 12.5</td>
<td>136.2 ± 5.7</td>
</tr>
<tr>
<td>dLEN melatonin</td>
<td>1.6 ± 0.1c (12.5 ± 0.5%)b, c</td>
<td>10.4 ± 0.4c</td>
<td>60.5 ± 3.7c</td>
<td>72.9 ± 9.2c</td>
</tr>
<tr>
<td>dLEN tamoxifen + melatonin</td>
<td>1.9 ± 0.6d (3.6 ± 0.5%)ab, c</td>
<td>2.0 ± 1.0a</td>
<td>22.7 ± 1.8a</td>
<td>46.8 ± 4.4a</td>
</tr>
</tbody>
</table>

NOTE: Values are mean ± SD (n = 3/group).
a,bThree animals (tumors)/group; ±SD tumor weights, in MCF-7 (ERα+) dLEN groups, vehicle treatment = 7.26 ± 0.22 g; tamoxifen treatment = 7.08 ± 0.22 g; dLEN melatonin treatment = 5.53 ± 0.20 g; tamoxifen + melatonin treatment = 1.95 ± 0.09 g, respectively. All tumors were harvested during the mid-dark phase at 24:00 hours. 
bValues in parenthesis expressed as % of arterial glucose supply. 
abP < 0.05 versus vehicle. 
bP < 0.05 versus tamoxifen. 
bP < 0.05 versus tamoxifen and melatonin.
ERK1/2, SRC, and FAK, all of which are activated by dLEN. STAT3, in turn, induces the expression of various genes involved in tumor promotion and progression, including Myc and cyclin D1 (43). Furthermore, activation of STAT3 promotes a feed-forward loop involving continuous production of the proinflammatory cytokine IL6 and activation of NF-κB (45). Supporting a report by Alvarez-Garcia and colleagues (46), analysis of tissue-isolated breast tumor xenografts from rats in a LD 12:12 lighting schedule showed a significant 80% reduction in IL6 mRNA expression at night as compared with those in dLEN (data not shown). Thus, the ability of melatonin to inhibit p-STAT3 levels in these breast cancer xenografts could result from either suppression of c-SRC/FAK phospho-activation, IL6 expression, or both, to inhibit cell survival, proliferation, metastasis, angiogenesis, and endocrine resistance.

Our studies also suggest that ERα phosphorylation and transactivation is circadian regulated by the nocturnal melatonin signal and that dLEN-induced melatonin suppression is associated with elevated phosphorylation of the ERα at S118 and S167 and possibly involved in tamoxifen resistance. These data combined with our reports (47) and that of others (48) that melatonin inhibits ERα transcriptional activity, and our unpublished data in cell lines that EGF or insulin—like growth factor-I stimulates phosphorylation of ERα at both Ser118 and Ser167, and that melatonin administration can inhibit this phosphorylation suggests that melatonin regulates ERα transcriptional activity through a phosphorylation-mediated mechanism.

In conclusion, the present investigation highlights and validates the importance of an intact endogenous nocturnal circadian melatonin signal in sensitizing human breast tumor cells to tamoxifen therapy. Given that nighttime melatonin significantly suppresses tumor kinase signaling; one could consider melatonin a broadly based ‘circadian-regulated kinase inhibitor’ (CRKI) that exhibits potent antimetabolic, -proliferative, and progression/metastatic activity in breast cancer. Moreover, our work demonstrates that a comprehensive understanding and maintenance of host/cancer circadian biology and the circadian-regulated nature of cancer metabolism and signaling are essential to derive the maximal efficacy from tamoxifen and possibly other endocrine therapies. In this regard, the maximal efficacy of such therapies would seem to be dependent on their optimal temporal administration in alignment with the circadian timing in LD entrained patients or with exogenous melatonin in patients with LEN-induced circadian/melatonin disruption. It is plausible that many, if not all, patients with breast cancer are likely to be subjected to various degrees of LEN and may be circadian/melatonin disrupted as a result of lack of sleep, and/or chronic late night shift work. Therefore, LEN may represent a unique and previously unappreciated risk factor that could account for some forms of intrinsic and possibly acquired tamoxifen resistance and may even lead to a shortened survival time and even a decreased survival rate.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Writing, review, and/or revision of the manuscript: R.T. Dauchy, S. Xiang, L. Mao, M.A. Wren, M. Anbalagan, A. Hauch, T. Frasch, B.G. Rowan, D.E. Blask, S.M. Hill
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R.T. Dauchy, L. Yuan, T. Frasch, S.M. Hill
Study supervision: R.T. Dauchy, D.E. Blask, S.M. Hill

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References


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