Melatonin uptake and growth prevention in rat hepatoma 7288CTC in response to dietary melatonin: melatonin receptor-mediated inhibition of tumor linoleic acid metabolism to the growth signaling molecule 13-hydroxyoctadecadienoic acid and the potential role of phytomelatonin

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Both physiological and pharmacological levels of the pineal hormone melatonin exhibit substantial anticancer activity in tissue-isolated rat hepatoma 7288CTC via melatonin receptor-mediated blockade of tumor uptake of linoleic acid (LA) and its metabolism to the mitogenic signaling molecule 13-hydroxyoctadecadienoic acid (13-HODE). Melatonin is also present in significant amounts in edible plants and is supplied in nutritional supplements. We confirmed the presence of significant quantities of melatonin in 20 varieties of edible plants. In pinealectomized tumour-free rats, 3 weeks of ingestion of either 5 or 50 ng/day of melatonin in a semi-purified diet resulted in a dose-dependent elevation in steady-state plasma melatonin levels within the nocturnal physiological range. In pinealectomized tumor-bearing rats, the daily intake of 5 µg/day of melatonin for 3 weeks resulted in an enhanced amplitude and duration of the nocturnal melatonin levels within physiological circulating limits. The nocturnal melatonin amplitude in rats ingesting 500 ng of melatonin/day remained within the physiological range. A dose-related increase in tumor concentrations of melatonin occurred in animals ingesting melatonin from the diet. Perfusion of tumors in situ with physiological, nocturnal blood levels of melatonin resulted in a mean 31% uptake and retention of the melatonin. Chronic ingestion of 50, 500 ng or 5 µg of melatonin/day supplied in a semi-purified 5% corn oil diet led to a significant dose-dependent reduction in the rates of tumor total fatty acid uptake, LA uptake, 13-HODE production and tumor growth. The co-ingestion of melatonin receptor antagonist S20928 completely blocked the effects and prevented the intra-tumoral accumulation of melatonin. Melatonin receptor-mediated suppression of tumor growth, LA uptake and metabolism, and stimulation of tumor melatonin uptake and retention in response to the dietary intake of phytomelatonin from edible plants or melatonin from nutritional supplements, could play an important role in cancer growth prevention.

Introduction

Many environmental factors influence the development and growth of cancer and it is known that diet exerts a major impact on all stages of oncogenesis. Several nutritional and epidemiological studies indicate that consuming diets rich in vegetables and fruits may help reduce the incidence of a broad range of malignancies (1,2). In this regard, plant foods provide a high level of antioxidant nutrients such as vitamins C, E and beta carotene. They also contain other phytochemicals with estrogenic or anti-estrogenic activity such as flavones, isoflavones, polyphenols, chalcones and indoleamines (3). With respect to plant-derived indoleamines, indole-3-carbinol (I3C) is a major glucosinolate found in cruciferous vegetables such as Brussels sprouts, cabbage, broccoli and cauliflower (4). I3C has been shown to inhibit rodent mammary carcinogenesis in vivo (5), human breast cancer growth in vitro via anti-estrogenic mechanisms (6), and human prostate cancer cell growth in vitro via anti-epidermal growth factor (EGF)-related mechanisms (7).

Melatonin (N-acetyl, 5-methoxytryptamine) is a chronobiotic, indoleamine neurohormone produced by the pineal gland during darkness in mammals, including humans (8). At both physiological and pharmacological blood concentrations, melatonin plays an important role in a number of physiological and pathophysiological processes including circadian rhythm regulation, sleep disturbances, seasonal reproduction, retinal physiology, immune function, intermediary metabolism (9) and tumorigenesis (10,11). Melatonin’s mechanism of action in the regulation of many of these processes is thought to involve inhibitory G protein-coupled melatonin receptors (MT1 and MT2) leading to the suppression of cAMP production, and a modulation in the activity of related signaling pathways as well (12). Melatonin is also a highly efficient and potent free radical scavenger/antioxidant molecule. Physiological blood levels of this indole are tightly correlated with the total antioxidant capacity of the serum (13,14).

In addition to its endogenous production by the pineal gland in vertebrate species, significant quantities of melatonin have been detected in all major non-metazoan taxa studied including bacteria, dinoflagellates, euglenoids, trypanosomids, fungi, rhodophyceans, phaeophyceans, chlorophyceans and angiosperms (15). Among angiosperms, melatonin is found in a number of species of fruits, vegetables and medicinal plants consumed by animals and humans. Melatonin is present in edible plants in concentrations that are several orders of magnitude higher than nocturnal blood concentrations produced by the pineal gland and it is found in all plant parts including roots, stems, leaves, fruit and seeds (15–22). Additionally, the consumption of melatonin-rich food acutely elevates immunoreactive (IR) and bioactive blood melatonin levels in chickens (17).

A more abundant source of exogenous melatonin in the human diet comes from commercially available nutritional supplements regularly consumed by millions of people throughout the world most often for sleep problems and/or...
jet lag (23,24). These supplements contain anywhere from 250 μg to 5 mg of melatonin delivered in either gel-cap or tablet form (24). In humans, the absolute oral bioavailability of milligram doses of oral melatonin from nutritional supplements is ~15% (25) whereas the oral bioavailability of microgram doses varies from 10 to 56%, presumably due to variations in first-pass hepatic metabolism (26). Nevertheless, the oral ingestion of microgram amounts of melatonin results in near physiological nocturnal blood levels of melatonin (27).

In comparison, nutritional supplements containing milligram amounts of melatonin can produce peak circulating melatonin levels that are 10-100 times greater than the normal peak produced by the pineal gland during darkness (25). Although the absolute bioavailability of a 300 μg oral dose of melatonin in male rats was reported to be 53% (28), the absolute oral bioavailability and physiological effects of melatonin derived from foodstuffs is unknown for any animal species (18). How-
cannulated, and blood samples were collected from each vessel simultaneously during the light phase (08:00 h). Measurements of arteriovenous (A-V) differences for total FAs, LA and 13-HODE were performed and rates of uptake and release were calculated as described previously (41–44). Two days prior to the termination of tumor growth study #1, blood collections (1 ml) were made via canula puncture during a 24-h period as described above. Steady-state plasma melatonin concentrations were measured by RIA as described below.

Effects of perfusion of tissue-isolated hepatoma 728SCTC in situ with different doses of melatonin on FA and LA uptake, 13-HODE release, DNA content and [3H]thymidine incorporation into DNA

Tumors weighing ~5 g in pinealectomized male Buffalo rats, were perfused in situ as described previously (40). Virtually melatonin-free arterial blood was collected during the early light phase (08:15–08:45 h) from pinealectomized donor male Sprague–Dawley rats. Donor blood was contained in a chilled (4 °C) reservoir and was pumped to the tumor through a warm water bath (37 °C) and an artificial lung as described previously (36,37). Upon the establishment of steady-state perfusion conditions, melatonin was added to the perfusate to achieve blood plasma concentrations of either 0, 100, 300 or 600 pm, 1 nM or 1 µM. Following 2 h of perfusion with melatonin, A-V differences for total FAs, LA, 13-HODE, hematoctrit, pO2, pCO2, and pH were measured as described previously (36,37,41–44). Incorporation of [3H]thymidine into tumor DNA was initiated 20 min before the end of the experiment by injection of 20 µl of physiological saline containing 2 µCi [methyl-3H]thymidine/g estimated tumor weight into the arterial catheter immediately prior to its entrance into the tumor. The [3H]thymidine made one pass through the tumor; unincorporated radioactivity was washed out of the perfused tumor during the remaining 20 min of perfusion. Radioactivity incorporated into tumor DNA was measured by liquid scintillation using internal standardization and is reported as d.p.m./mg tumor DNA (56,40,43). Tumor DNA content was measured in 20% (w/w) homogenates fluorometrically using Hoechst dye 33258 using the procedure described in Technical Bulletin #119, Hoefer Scientific Instruments (San Francisco, CA).

Lipid extraction and analyses

Total plasma, tumor and dietary lipids were extracted using the procedure of Folch et al. (50) and analyzed by gas chromatography, as described previously (40,43,44). Extraction of 13-HODE from tumor venous blood and analysis by HPLC were performed as described (40,43,44).

Melatonin analysis

Melatonin analyses were carried out on extracted plant tissue samples, arterial and venous blood plasma samples and tumor tissue samples using a highly sensitive RIA purchased from ALPCO (Windham, NH).

Statistical analysis

Differences among mean latencies to tumor onset, tumor total FA and LA uptake, tumor 13-HODE release, tumor melatonin and FA concentrations and plasma melatonin levels were determined with a one-way ANOVA followed by Student-Newman-Keul’s multiple comparisons post hoc test. Differences among the slopes of regression lines between experimental groups and their corresponding control groups were determined by regression analyses and tests for parallelism. Differences were considered to be statistically significant at P < 0.05.

Materials

Chloroform (HPLC grade), methanol, glacial acetic acid, heptane, hexane and Sep-Pak C18 Cartridges for HPLC sample extraction were purchased from Fisher Chemical (Pittsburg, PA). Methly esters of rapeseed oil and FA HPLC standards were obtained from Supelco (Bellefonte, PA). Free FA and lactic acid standards, BHT, boron trifluoride-methanol, enzymes, nucleotides and buffers were purchased from Sigma Chemical (St Louis, MO). The HPLC standards, 5-HETE and 13-HODE (each as the racemic mixture) were purchased from Cayman Chemical (Ann Arbor, MI). Dietary ingredients were purchased from US Biochemicals (Cleveland, OH). The melatonin RIA kit was purchased from ALPCO.

Results

IR-melatonin concentrations in edible plants and plant parts

Based on a preliminary screening of levels of IR-melatonin in 41 edible plants and plant-derived foods (data not shown), we determined the plant to plant and within plant melatonin concentrations in a variety of 20 edible plants and plant parts including some of those reported previously to contain significant amounts of melatonin (16–20,51–53). In a few cases, melatonin concentrations were assessed in different parts of the same plant. This analysis was performed with three different extraction procedures that utilized either PBS, methanol or chloroform. Since significantly more IR-melatonin/g dry plant tissue was extracted with PBS than with either methanol or chloroform (data not shown), results obtained only with PBS extraction are reported. Table I shows that different amounts of melatonin were extracted from different plants or plant parts. Peanuts, walnuts and mustard seed contained the highest mean IR-melatonin levels (i.e. >1 ng/g) with mustard seed containing the highest amounts (i.e. 1.9 ng/g). The lowest mean quantities of IR-melatonin (i.e. <20 pg/g) were found in grapes. There was considerable variation in IR-melatonin levels between and within plant species.

Effects of dietary melatonin on 24-h steady-state plasma melatonin concentrations in pinealectomized rats

To determine whether the dietary intake of melatonin, in quantities approximating those supplied by edible plants, would raise nocturnal circulating melatonin concentrations

<table>
<thead>
<tr>
<th>Plant type</th>
<th>MLT (pg/g dry tissue)</th>
<th>Plant type</th>
<th>MLT (pg/g dry tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rolled oats</td>
<td>791 ± 7.2 (712–887)</td>
<td>Red grape (whole)</td>
<td>19.4 ± 5.8 (13–26)</td>
</tr>
<tr>
<td>Asparagus</td>
<td>766 ± 2.6 (532–1028)</td>
<td>Red grape (skin)</td>
<td>14.2 ± 2.3 (12–18)</td>
</tr>
<tr>
<td>Fresh mint</td>
<td>496.6 ± 116.3 (360–609)</td>
<td>Red grape (Pulp)</td>
<td>22.7 ± 2.5 (21–26)</td>
</tr>
<tr>
<td>Broccoli</td>
<td>266.7 ± 168.1 (112–425)</td>
<td>Green tea</td>
<td>92.0 ± 11.1 (78–104)</td>
</tr>
<tr>
<td>Mustard seed</td>
<td>1913.3 ± 310.9 (1512–2198)</td>
<td>Black tea</td>
<td>405.0 ± 84.3 (332–489)</td>
</tr>
<tr>
<td>Tomatoes</td>
<td>539.3 ± 58.1 (464–602)</td>
<td>Green olives</td>
<td>83.6 ± 9.1 (75–92)</td>
</tr>
<tr>
<td>St John’s wort</td>
<td>196.1 ± 58.9 (140–258)</td>
<td>Black olives</td>
<td>89.4 ± 16.9 (66–106)</td>
</tr>
<tr>
<td>Angelica</td>
<td>251.2 ± 45.6 (209–312)</td>
<td>Peanuts</td>
<td>1167.0 ± 1199.2 (128–2312)</td>
</tr>
<tr>
<td>Walnuts</td>
<td>1497.7 ± 627.6 (907–2008)</td>
<td>Concord grape (whole)</td>
<td>17.1 ± 5.4 (9–21)</td>
</tr>
<tr>
<td>Sunflower seeds</td>
<td>42.6 ± 7.9 (31–50)</td>
<td>Concord grape (skin)</td>
<td>32.4 ± 1.9 (30–33)</td>
</tr>
<tr>
<td>Cucumber</td>
<td>59.3 ± 3.1 (24–94)</td>
<td>Concord grape (pulp)</td>
<td>19.2 ± 2.7 (16–22)</td>
</tr>
<tr>
<td>Brussels sprouts</td>
<td>168.8 ± 18.1 (145–188)</td>
<td>Sweetcorn</td>
<td>32.2 ± 16.7 (7–42)</td>
</tr>
<tr>
<td>All plants</td>
<td>374 ± 513 (137%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Foods were either locally grown or were purchased at a local supermarket. A sample of each plant was removed, freeze-dried and a portion (0.25 g) subsequently extracted with 1× PBS. The supernatants were analyzed for their melatonin content using an ultrasensitive double antibody RIA kit. Assay sensitivity was 0.3 pg/ml. Values represent the mean ± SD melatonin levels (pg/g dry plant material). Four different samples were analyzed per plant type. Numbers in ( ) represent the range of melatonin values within a given plant type; numbers in { } represent the within plant % coefficient of variation (CV); numbers in [ ] represent the between plant % CV.
into the nocturnal physiological and/or pharmacological ranges in the absence of the endogenous melatonin signal we consulted recommendations by the American Institute for Cancer Research for an ‘all-plant’ diet (3) as well as values of edible plant levels of melatonin obtained from the literature (16–20,51–53) and the present study. From this information, an estimate was made that the daily human consumption of a ‘melatonin-rich’ plant diet would supply ~500 ng of melatonin/day. Therefore, pinealectomized (i.e. no endogenous melatonin signal) non-tumor-bearing rats were provided with a semi-purified diet ad libitum supplemented with melatonin such that rats consumed either 0 ng, 500 ng, 5 μg or 50 μg/day over a 3-week period. Rats were maintained on a 12L:12D light:dark cycle (lights on 06:00–18:00 h). Melatonin levels in the 50 or 5 μg/day melatonin groups versus 500 ng/day melatonin and control groups at all time points, \( P < 0.05 \); melatonin levels in the 5 μg/day melatonin group at 04:00 h versus all other time points in the same group, \( P < 0.05 \).

![Graph of plasma melatonin levels](image)

**Fig. 1.** Effects of dietary melatonin on mean (±SD) plasma levels of melatonin in tumor-free pinealectomized male rats. Rats were fed a semi-purified diet (containing 10% corn oil) containing melatonin so that rats would ingest either 0 ng, 500 ng, 5 μg or 50 μg/day over a period of 3 weeks \((n = 6 \text{ rats/group})\). At the end of this period, blood samples were obtained around-the-clock via cardiac puncture at six circadian time points. Rats were maintained on a 12L:12D light:dark cycle (lights on 06:00–18:00 h). Melatonin levels in the 50 or 5 μg/day melatonin groups versus 500 ng/day melatonin and control groups at all time points, \( P < 0.05 \); melatonin levels in the 5 μg/day melatonin group at 04:00 h versus all other time points in the same group, \( P < 0.05 \).

Tumor growth study #1: effects of dietary melatonin intake on the growth, LA uptake, and 13-HODE production in hepatoma 7288CTC and steady-state plasma (24-h) and tumor melatonin concentrations in pineal-intact rats

In tumor-bearing, pineal-intact rats ingesting the 500 ng melatonin/day diet, the latency to tumor (i.e. palpable tumors) onset \((13.3 ± 1.3 \text{ days post-implant})\) was significantly later \((P < 0.05)\) than in controls \((8.0 ± 2.2 \text{ days post-implant})\). The tumor growth rate \((0.32 ± 0.02 \text{ g/day})\) was markedly slower than in the control group ingesting a melatonin-free diet \((0.76 ± 0.03 \text{ g/day})\) (Figure 2). In the group ingesting the 5 μg melatonin/day diet, the latency to tumor onset \((20.8 ± 2.1 \text{ days post-implant})\) was substantially extended \((P < 0.05)\) as compared with both the 500 ng melatonin/day and control groups. The tumor growth rate in the 5 μg melatonin/day group \((0.18 ± 0.02 \text{ g/day})\) was much slower \((P < 0.05)\) than that in the 500 ng melatonin/day and control groups (Figure 2).

A-V difference measurements were performed across tumors at the end of the growth period in each group are shown in Figure 2. These measurements indicated that tumor LA and total FA uptakes (Table II), as a percent of supply, were significantly reduced in the 5 μg and 500 ng melatonin/day groups as compared with the control group. In comparison with the 500 ng melatonin/day group, tumor LA and total FA uptakes in the 5 μg melatonin/day group were substantially less (Table II). Similarly, the tumor concentrations of LA and total FA in the 5 μg/day group were significantly diminished as compared with those in the control group (Table II). Tumor LA and total FA concentrations in 500 ng melatonin/day group were considerably less than in the controls. Additionally, tumor LA and total FA concentrations were significantly less in the 5 μg melatonin/day group as compared with animals ingesting 500 ng melatonin/day. The dietary intake of 5 μg or 500 ng melatonin/day resulted in tumor 13-HODE release that was markedly less than in the controls (Table II). Tumor 13-HODE release was also significantly lower in the rats ingesting 5 μg versus 500 ng melatonin/day.

Figure 3 shows that a robust nocturnal plasma melatonin signal was present in the tumor-bearing, pineal-intact rat
groups depicted in Figure 2 that ingested either 0 or 500 ng melatonin/day. However, in animals ingesting 500 ng melatonin/day, peak nocturnal levels of melatonin at 24:00 and 04:00 h, albeit still within the physiological range, were ~50% lower than in control rats. In animals ingesting 5 μg of melatonin/day, circulating melatonin remained consistently at higher levels than in the physiological range, and in the other two groups, throughout the 24-h period with no rhythm being detected. Table III shows that tumor concentrations of melatonin were ~10- and 400-fold higher in the 500 ng and 5 μg of melatonin/day groups, respectively, than in the non-supplemented control group.

**Dose-response effects of melatonin on LA and total FA uptake, 13-HODE release, DNA content and [3H]thymidine incorporation into DNA and melatonin uptake in hepatoma 7288CTC perfused in situ pineal-intact rats**

Figure 4A shows the effects of the perfusion of tumors in situ, with whole blood from pinealectomized donor rats containing plasma concentrations of added melatonin ranging from 0 to 1 μM, on tumor LA and total FA uptake and 13-HODE release. There was a significant dose-dependent suppression of tumor LA and total FA uptake and 13-HODE release that reached saturation at 1 nM melatonin. A Dixon plot (54) of the dose-response data for melatonin inhibition of the rate of total FA uptake (Figure 4A, inset) revealed that the $K_i$, calculated for suppression of total FA uptake was 93 pM, which represents the concentration of melatonin that inhibited FA uptake by $\frac{1}{2}V_{max}$. Figure 4B demonstrates a similar dose-dependent suppression of tumor DNA content and [3H]thymidine incorporation into DNA by melatonin. As with FA uptake and 13-HODE release, this inhibition of cell proliferation reached saturation at 1 nM melatonin. A-V difference measurements made across tumors perfused with various concentrations of melatonin revealed a tumor melatonin uptake and retention, as a function of supply, that ranged from 20 to 45%; across all melatonin concentrations tested, mean (±SD) tumor melatonin uptake was 31.3 ± 20.4%.

**Tumor growth study #2: effects of the dietary intake of melatonin and melatonin receptor antagonist S20928 on the growth, LA uptake, 13-HODE production and melatonin concentration in hepatoma 7288CTC in pineal-intact rats**

As in our initial experiment, in rats ingesting the 500 ng melatonin/day in the diet, the latency to tumor onset (17 days post-implant) was substantially later ($P < 0.05$) than in the control group (10 days post-implant). The tumor growth rate (0.19 ± 0.03 g/day) was much slower than in the control group consuming a melatonin-free diet (0.55 ± 0.06 g/day) (Figure 5). These effects were completely prevented in the group receiving melatonin receptor antagonist S20928 (5 μg/day) in conjunction with melatonin (500 ng/day). In the group ingesting S20928 alone in the diet, the appearance of tumors (8 ± 0 days post-implant) was sooner than in the controls whereas the tumor growth rate was increased by 65%. While latency to tumor onset (11 days post-implant) was only 24 h later than the controls, the tumor growth rate in the rats ingesting 50 ng of melatonin/day was significantly slower than in the controls (Figure 5).
Table III. Effects of dietary melatonin intake (0, 50 and 500 ng/day) either alone or in combination with melatonin receptor antagonist S20928 (5 μg/day) on mean (±SD) total FA uptake, FA levels, LA uptake, LA levels, 13-HODE release and melatonin levels in tissue-isolated rat hepatoma 7288CTC in male rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total FA uptake (μg/min/g) [%]</th>
<th>Tumor FA levels (μg/g)</th>
<th>LA uptake (μg/min/g) [%]</th>
<th>Tumor LA levels (mg/g)</th>
<th>13-HODE release (ng/min/g)</th>
<th>Melatonin levels (pg/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (5% CO)</td>
<td>7.3 ± 2.4 [39.3 ± 4.1]</td>
<td>34.3 ± 2.2</td>
<td>1.5 ± 0.4 [40.0 ± 3.4]</td>
<td>7.4 ± 0.8</td>
<td>38.7 ± 4.3</td>
<td>3.2 ± 0.4</td>
</tr>
<tr>
<td>Melatonin (50 ng/day)</td>
<td>6.8 ± 0.7 [31.3 ± 3.9]</td>
<td>27.9 ± 1.0</td>
<td>1.7 ± 0.3 [32.6 ± 3.4]</td>
<td>4.4 ± 0.2</td>
<td>33.7 ± 3.5</td>
<td>95.1 ± 10.1</td>
</tr>
<tr>
<td>Melatonin (500 ng/day)</td>
<td>5.6 ± 1.1 [20.3 ± 3.3]</td>
<td>15.3 ± 1.6</td>
<td>1.2 ± 0.6 [16.3 ± 6.8]</td>
<td>2.4 ± 0.2</td>
<td>21.8 ± 4.8</td>
<td>406.4 ± 88.0</td>
</tr>
<tr>
<td>Melatonin (500 ng/day) + S20928 (5 μg/day)</td>
<td>7.7 ± 1.1 [37.9 ± 2.8]</td>
<td>38.0 ± 1.9</td>
<td>1.6 ± 0.5 [39.6 ± 5.2]</td>
<td>8.3 ± 1.7</td>
<td>44.2 ± 3.3</td>
<td>12.0 ± 13.9</td>
</tr>
<tr>
<td>S20928 (5 μg/day)</td>
<td>7.9 ± 1.0 [45.4 ± 3.1]</td>
<td>45.1 ± 2.6</td>
<td>1.8 ± 0.5 [47.8 ± 7.1]</td>
<td>11.1 ± 1.9</td>
<td>47.0 ± 4.4</td>
<td>13.1 ± 5.7</td>
</tr>
</tbody>
</table>

Biochemical parameters were determined at the end of the growth period for each of the treatment groups in tumor growth study 2 (see Figure 5). Melatonin and/or S20928 were contained in a semi-purified 5% corn oil (CO) diet. Diets were started 2 weeks prior to tumor implantation and were continued until the end of the growth study. [%] represents percent uptake as a function of FA or LA supply. *P < 0.05 versus control, melatonin + S20928. **P < 0.05 versus control, melatonin (500 ng/day). ***P < 0.05 versus control; n = 6 rats (tumors)/group.

Fig. 4. Dose-response effects of increasing concentrations of melatonin on total FA and LA uptakes, 13-HODE release (A), DNA content and [3H]thymidine incorporation into DNA (B) in rat hepatoma 7288CTC perfused in situ. Tumors were perfused over a 2 h period with whole blood, from pinealectomized donor rats (no circulating melatonin), to which melatonin was added to achieve final concentrations of either 100, 300 and 600 μM, 1 nM or 1 μM. The inset (A) is a Dixon plot representing reciprocal rates of total FA uptake as a function of the plasma melatonin concentration; Kᵢ for suppression of total FA uptake = 93 μM. Tumors were perfused with [3H]thymidine during the final 20 min of the perfusion period. Three tumors were perfused in each treatment group. (A) FA and LA uptakes and total FA uptake in each group shown in Figure 5 indicated that tumor FA and LA uptakes (Table III), as a percent of supply, were significantly reduced in both the 500 and 50 ng of melatonin/day groups as compared with the control group. In comparison with the 50 ng of melatonin/day group, tumor total FA and LA uptakes in the 500 ng melatonin/day group were much less. Additionally, the tumor concentrations of total FA and LA in the 500 ng melatonin/day group were significantly reduced as compared with the control group (Table III). In the 50 ng of melatonin/day group, tumor total FA and LA concentrations were significantly less in the controls. Tumor LA and total FA concentrations in the 500 ng of melatonin/group were significantly less than in the animals that consumed 50 ng of melatonin/day. The dietary intake of 50 and 500 ng of melatonin/day resulted in tumor 13-HODE release that was significantly lower than in the controls (Table III). Tumor 13-HODE release was also 35% lower in the rats ingesting 500 versus 50 ng of melatonin/day. Tumor melatonin concentrations in the 50 and 500 ng of
melatonin/day group were increased by 30- and 127-fold, respectively, over concentrations in the control tumors (Table III). As compared with the 50 ng of melatonin/day group, tumor melatonin concentrations in the 500 ng of melatonin/day group were 4-fold higher.

When rats ingested 500 ng melatonin/day in combination with melatonin receptor antagonist S20928, the suppressive effects of melatonin on tumor FA and LA uptake, 13-HODE release, FA and LA concentrations, and melatonin concentrations were completely prevented (Table III). Interestingly, the ingestion of S20928 by itself caused significant increases in tumor total FA and LA uptakes, FA and LA concentrations and 13-HODE release relative to controls. Although the tumor melatonin concentration was markedly lower in the group ingesting S20928 alone, it was still 4-fold higher than in the control group (Table III).

Discussion

We confirmed the presence of significant quantities of IR-melatonin reported previously to be present in a wide variety of edible plant species (15-22). We also documented the presence of significant amounts of IR-melatonin in a number of other edible plants and plant parts that had not been examined previously. All plant species analyzed contained detectable levels of IR-melatonin and several plant foods with the highest levels, including peanuts, walnuts and mustard seed contained >1 ng of melatonin/g of dry tissue weight. Although the amounts of IR-melatonin measured in mustard seed were the highest of all plants analyzed, they were considerably lower than the quantities of melatonin found in these same plant species as reported by other investigators (51,52).

Due to its physicochemical properties, melatonin is very difficult to isolate from plant samples due to the presence of melatonin-binding proteins, carbohydrates, lipids, pigments and other unidentified plant components in the plant tissue may have interfered or cross-reacted with the melatonin RIA (19,20). Therefore, it is likely that the amounts of IR-melatonin detected here may represent either over- or underestimated of the amounts than would have actually been detected had more sensitive and accurate methods, such as gas chromatography-mass spectroscopy, been available (19,20). Despite these potential problems in measuring plant melatonin levels with RIA, our results provide additional important evidence that this indoleamine is present in non-trivial concentrations in edible foodstuffs and justifies its designation as ‘phytomelatonin’.

As alluded to above, pharmacological amounts of melatonin or a melatonin agonist delivered through dietary means has been successfully used in rodent species as a strategy to gradually phase advance central circadian pacemaker activity by reinforcing the endogenous melatonin signal itself as well as to enhance the sensitivity of the circadian timing system to phase-shifting stimuli (29,30). These observations indicate that sufficient amounts of melatonin are absorbed and delivered to melatonin-sensitive target tissues to effect changes in circadian physiology. During a 3-week period in the present study, tumor-free, pinealectomized rats consumed a semi-purified diet containing amounts of melatonin that ostensibly would be present in a human phytomelatonin-rich diet. As expected in pinealectomized control rats, the endogenous nocturnal circadian melatonin signal was completely absent. Also, in pinealectomized rats ingesting 5 µg of melatonin/day, a ‘quasi’-physiological nocturnal melatonin signal was reconstituted because the circadian, nocturnal feeding rhythm remained intact in these animals. However, the peak amplitude of the rhythm was phase-delayed by 4 h from the nocturnal peak that is normally generated endogenously by the pineal gland. Following 3 weeks of 50 µg of melatonin/day, melatonin levels were constantly elevated in the nocturnal physiological range throughout the 24-h period. Surprisingly, however, 500 ng of melatonin ingested each day for 3 weeks did not, however, increase blood levels of melatonin above those observed in the pinealectomized controls.

The lack of elevated blood melatonin levels in pinealectomized rats ingesting 500 ng of melatonin/day may, at first, seem counterintuitive; however, there is a plausible explanation for this apparent paradox. It is important to note that the direct, intravenous infusion of 500 ng or more of melatonin/ml plasma/h for 2 h is required to produce a physiological, ‘nocturnal-type’ elevation in plasma melatonin concentrations in the rat during the light phase (55). Infusion with any less than this amount has no effect on plasma melatonin concentrations, which remain at low daytime levels. Therefore, it has been proposed that a significant, nocturnal-type rise in circulating melatonin levels only occurs when the melatonin supply reaches a critical threshold level that exceeds the metabolizing capacity of the liver (55). Interestingly, it has been estimated that about one-third of the melatonin released by the pineal gland and/or extra-pineal sources during the dark phase is removed by an unknown mechanism other than hepatic metabolism (56). In fact, ~25% of the melatonin infused into rats accumulates in the gut, which contains over 90% of the whole body melatonin content (57). Not only is the gut an extra-pineal site of melatonin synthesis, but it also serves as an important ‘sink’ for circulating melatonin (56,57). Other tissues that sequester melatonin also include the liver, colon, muscle, kidney, adrenal and pituitary glands. Once melatonin has entered these tissues, it becomes bound to tissue binding proteins (56). Thus, in the present study, the hepatic extraction, renal clearance and tissue uptake and retention of melatonin may have been greater in pinealectomized than in pineal-intact animals. The fact that peak nocturnal plasma melatonin levels were 5-fold greater and of much longer duration in pineal-intact animals ingesting 5 µg of melatonin/day than in pinealectomized animals ingesting the same amount of the indoleamine supports this notion. If this postulate is correct, it not only could partially explain the low plasma melatonin levels in the 500 ng of melatonin/day group but also the nocturnal physiological melatonin titers in rats consuming microgram quantities of dietary melatonin. Therefore, these plasma melatonin profiles, represented here as a ‘snap-shot’ in time in relation to dietary melatonin ingestion, may not accurately reflect the true nature of the dynamics of melatonin’s distribution to peripheral target tissues.

Dietary melatonin intake, including quantities of the indole that were estimated to be consumed by humans from a phytomelatonin-rich diet, was implemented 2 weeks prior to tumor implantation and continued for several weeks thereafter. This cancer prevention strategy markedly delayed tumor onset and diminished the high rates of hepatoma growth, LA uptake and 13-HODE formation, in a dose-dependent manner. Since 13-HODE is inactivated by UDP-glucuronosyltransferases (UGT) in human liver (58), it is possible that low levels of these enzymes are expressed in liver tumors. Although speculative,
up-regulation of UGTs may represent another mechanism by which 13-HODE levels in rat hepatoma 7288CTC are suppressed by phytomelatonin. Perfusion of tumors in situ with melatonin for only 2 h at concentrations corresponding to physiological nocturnal plasma levels demonstrated that as little as 93 pM and as much as 1 nM of melatonin were effective in suppressing tumor LA uptake, 13-HODE production and cell proliferation. That these inhibitions required melatonin receptors was strongly indicated by the ability of melatonin receptor antagonist S20928, when co-ingested in the diet, to completely block the effects of melatonin as observed in our previous melatonin perfusion studies (36). A most interesting discovery was that accelerated rates of tumor growth and LA uptake and metabolism in rats consuming dietary S20928 alone duplicated these same effects induced by pinealectomy or exposure to either constant light or dim light during darkness (36,59). These novel results are the first to show, in the area of melatonin/cancer biology, that interference with the action of the endogenous circadian melatonin signal, at the receptor level, was tantamount to eliminating the melatonin signal itself at its source in the pineal gland.

An unexpected finding was the dose-related accumulation of melatonin in tumor tissue following several weeks of dietary melatonin intake. In fact, tumors perfused in situ for only 2 h with a range of blood plasma melatonin levels encompassing the physiological to pharmacological ranges exhibited a robust 31% uptake and retention of melatonin as a function of supply. Thus, over the course of tumorigenesis, the daily occurrence of tumor melatonin uptake during every 12-h dark period could reasonably explain the substantial accumulation of melatonin within tumor tissue over the course of 3–6 weeks. Whether cancer cells per se and/or other cell types present in tumors were responsible for melatonin uptake is unknown. However, when MCF-7 human breast cancer cells were exposed to 1 nM melatonin in culture over a 3-day period, cellular melatonin uptake was ~50% of the melatonin available in the incubation medium (D.E.Blask, S.T.Wilson and G.M.Vaughan, unpublished results). In this regard, it has been demonstrated that the vast majority of human invasive ductal breast carcinomas not only express MT1 melatonin receptors (60) but also contain melatonin levels that are 1000-fold higher than nocturnal serum levels (61). Similarly, high levels of melatonin have been also reported in non-neoplastic breast tissue, including adipose tissue, from both healthy subjects or breast cancer patients (61). The considerable tumor uptake and retention of melatonin observed in the present investigation indicated that, like the gut and other tissues (56,57), tissue-isolated hepatoma 7288CTC was an important repository for melatonin supplied in the diet. Thus, the inability of nocturnal plasma melatonin levels to exceed the physiological range in tumor-bearing rats ingesting melatonin may be ascribed, in part, to rapid tumor sequestration of circulating melatonin. As a result of this process occurring repeatedly over a period of several weeks, it is likely that these steady-state circulating melatonin levels reflect an equilibrium that had been reached between endogenous pineal melatonin production in combination with exogenous melatonin supplementation, on the one hand, and the processes of melatonin metabolism, clearance and uptake/retenion by normal and tumor tissues, on the other.

Normal rat liver cells express melatonin receptors of the MT2 subtype (62,63), while both MT1 and MT2 melatonin receptors are expressed in hepatoma 7288CTC (D.E.Blask, R.T.Dauchy, J.A.Krause, L.A.Sauer and F.Zalatan, unpublished results and ref. 64) and mediate melatonin-induced inhibition of FA uptake and 13-HODE production (36). Although the exact mechanism mediating melatonin’s uptake and retention by hepatoma 7288CTC is unclear, it also appears to be consistent with a receptor-mediated phenomenon as revealed by the blockade of tumor melatonin uptake by S20928 (65). Interestingly, both in rats ingesting S20928 either alone or in combination with dietary melatonin, tumor melatonin levels were 4-fold higher than in control tumors. It appears that while the vast majority of the melatonin available to the tumor tissue is prevented from being taken up by S20928, a much smaller but significant amount of melatonin still gains access to tumor tissue probably owing to melatonin’s highly lipophilic nature (8). For unknown reasons, the dynamics of this process must be different in control tumors in which no receptor blockade is present and only endogenous circulating melatonin levels are available. Although speculative at this time, intratumoral melatonin levels may ultimately reflect a balance between a combination of receptor-mediated uptake and diffusion of melatonin and its intracellular metabolism. Futhermore, the higher tumor FA and melatonin levels in rats ingesting S20928 alone suggest that any endogenous circulating melatonin that has accumulated within tumor tissue, probably via diffusion, may reside in an intracellular compartment that makes it unavailable for interaction with membrane-associated melatonin receptors and thus suppression of FA uptake.

Without direct evidence, a probable explanation for the majority of tumor uptake and retention of melatonin is desensitization and internalization of ligand-occupied melatonin receptors at the cellular level. As mentioned above, diffusion of melatonin into tumor tissue and cells appears to be a much smaller component of the overall mechanism. Once within the cancer cell, melatonin may then become uncoupled from its receptors at the cellular level. As mentioned above, diffusion of melatonin into tumor tissue and cells appears to be a much smaller component of the overall mechanism. Once within the cancer cell, melatonin may then become uncoupled from its receptors at the cellular level. As mentioned above, diffusion of melatonin into tumor tissue and cells appears to be a much smaller component of the overall mechanism. Once within the cancer cell, melatonin may then become uncoupled from its receptors at the cellular level. 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As mentioned above, diffusion of melatonin into tumor tissue and cells appears to be a much smaller component of the overall mechanism. Once within the cancer cell, melatonin may then become uncoupled from its receptors at the cellular level.
exogenous dietary sources as well as from endogenous fat stores (37). Based on the evidence presented here, a strong experimental case can be made that, like other indoleamine phytochemicals such as 13C, phytomelatonin or melatonin contained in nutritional supplements could have a potentially significant impact as a new strategy in human cancer prevention. In this regard, Reiter and colleagues (21) have proposed recently a provocative but cogent argument that melatonin may now have to be considered not only as a hormone, but as an antioxidant nutrient and/or vitamin as well.

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References


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