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The Influence of 1.2 μT, 60 Hz Magnetic Fields on Melatonin- and Tamoxifen-Induced Inhibition of MCF-7 Cell Growth

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We independently examined the findings of Harland and Liburdy, who reported that 1.2 μT_{rms}, 60 Hz magnetic fields could significantly reduce the inhibitory action of physiological levels of melatonin (10^{-9} M) and of pharmacological levels of tamoxifen (10^{-7} M) on the growth of MCF-7 human breast cancer cells in vitro. We used two testing protocols. In the melatonin study, the cell numbers per dish on day 7 of treatment were determined using a hemocytometer assay. In the tamoxifen study we used an expanded protocol, employing an alternative cell counting assay to characterize the cell numbers per dish on days 4, 5, 6, and 7. In both the melatonin and tamoxifen studies, cells were plated on 35 mm dishes and placed in each of two exposure chambers inside 5% CO_2 incubators. One exposure chamber was energized to produce 1.2 μT_{rms}, 60 Hz magnetic fields and the other chamber was not energized. Treatment was continuous until assays were performed. Cells were harvested at selected times, and enumerated without knowledge of treatment. In the melatonin study, the experiment was repeated three times, whereas in the tamoxifen study, each experiment was repeated nine times. In the melatonin study, cell numbers per dish were significantly reduced (by 16.7%) in the melatonin treated cultures after 7 days of incubation compared to control cultures, whereas in the presence of 1.2 μT_{rms}, 60 Hz magnetic fields, the melatonin treated cultures had the same cell populations as the control cultures. In the tamoxifen study, tamoxifen reduced the cell growth by 18.6 and 25% on days 6 and 7, respectively, in the chamber not energized, while in 1.2 μT_{rms}, 60 Hz fields, tamoxifen reduced the cell growth only by 8.7 and 13.1%, respectively. These results are consistent with those reported by Harland and Liburdy. A critical element of this successful replication effort was the constructive communication established and maintained with the original investigators. Bioelectromagnetics 22:122–128, 2001. Published 2001 Wiley-Liss, Inc.†

Key words: breast cancer; in vitro; ELF; replication study

INTRODUCTION

One important criterion for broad acceptance of low intensity, magnetic field induced biological effects is independent experimental confirmation of the original findings in other laboratories. The study reported here successfully confirms earlier reports of magnetic field influences on the growth of human breast cancer cells in vitro.

Melatonin (MEL) is a hormone whose nocturnal release may affect virtually every cell in the body; yet this nightly release decreases with adult age [Reiter, 1992, 1994]. In addition to the influence of MEL on ultradian rhythms, MEL has oncostatic properties [Tamarkin et al., 1981; Lissoni et al., 1991; Blask 1993). For example, the action of the chemical carcinogen dimethylbenzanthracene (DMBA) to produce mammary tumors in rats is hindered by MEL [Tamarkin et al., 1981]. This result was extended to human cells in a report that physiological levels of MEL reduce the growth rate of MCF-7 human breast cancer cells in vitro [Hill and Blask, 1988]. Liburdy and collaborators [Liburdy et al., 1993a,b; 1994] reproduced the growth inhibition caused by MEL in MCF-7 cells and also found that 1.2 μT_{rms}, 60 Hz sinusoidal magnetic fields (MF) could block that MEL-induced growth inhibition.

Tamoxifen (TMX) is used clinically to manage estrogen-positive breast cancer. TMX competitively binds to estrogen receptors and reduces estrogen-

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induced proliferation stimuli in breast cancer cells [MacGregor and Jordan, 1998]. The Liburdy laboratory extended their studies of the MCF-7 cell system by confirming that pharmacological levels (10^{-7} M) of TMX had inhibitory action on the growth of MCF-7 cells and demonstrating that MF could partially remove that inhibitory action [Harland and Liburdy, 1997].

This report describes our attempts to confirm both of the findings by Liburdy et al. Our MEL study was more limited and focused than our TMX study because of time and resource constraints.

MATERIALS AND METHODS

Magnetic Field Exposure

The 1.2 μT_{rms}, 60 Hz, sinusoidal magnetic fields (MF) were generated by an arbitrary waveform generator (Wavetek, Model 75, San Diego, CA) energizing a coil system. Each coil system consisted of two coils, containing 1000 turns of enameled wire in a 20 cm diameter coil, arranged in a Helmholtz configuration with the coils separated by 10 cm and oriented to produce a vertical magnetic field. Cell culture dishes were placed in the uniform field region between the coils for treatment with MF. Currents in the coils were adjusted and monitored using a multimeter (Fluke, model 8060A, Everett, WA) after the flux density was established with a fluxgate magnetometer (Bartington, model MAG-03, GMW Assoc., Redwood City, CA). This system has been described previously [Blackman et al., 1993].

The Helmholtz coil systems were mounted near the center of ventilated, magnetically shielded (Mu metal) boxes (Amuneal Corp., Philadelphia, PA) housed in 5% CO_2 incubators (Forma, models 3158 and 3326, Marietta, OH) maintained at 37°C. Ambient oscillating fields at the sample locations were thus reduced to under 0.04 μT_{rms}, and the ambient static field was reduced to less than 0.3 μT. Each incubator contained a coil system and shielding box, but only one set was energized for each experiment.

Cell Culture

The human breast cancer cells (MCF-7) were a generous gift from the Liburdy laboratory. The cells were grown at 37°C in an atmosphere of 5% CO_2 in DMEM (Sigma Corp., St. Louis, MO), supplemented with 10% fetal bovine serum (product 101, lot 10786, Tissue Culture Biologicals, Tulare, CA), which is the same source and lot number as used by Liburdy's laboratory. The medium also contained 100 U/ml penicillin and 100 mg/ml streptomycin. The protocols established by Liburdy's laboratory were essentially followed for all operations, including cell transfer and plating in 35 mm culture dishes. One exception to the protocol was that cells were grown in an incubator [Forma, model 3327) which had ambient 60 Hz magnetic fields of less than 0.1 μT_{rms} and a static magnetic field of 24.9 μT at an inclination of 65.9°N. Melatonin (*N*-acetyl-5-methoxy-tryptamine; Sigma, product M5250) was dissolved in 0.5 ml 95% ethanol at 10^{-2} M and further diluted in the growth medium to 10^{-9} M. Tamoxifen (Sigma, product T9262) was prepared in ethanol at 10^{-2} M and diluted to 0.25×10^{-7} M in complete medium for application to the cells. No difference in growth was observed for cells treated with the ethanol vehicle alone compared to untreated cells.

Treatment of Cells

In the MEL study, three replications of each test were performed, and each test employed a newly thawed sample of MCF-7 cells. MCF-7 cells were plated at either 2.5 or 7.5×10^4 cells/dish on each of nine dishes of 35 mm diameter. The cells were allowed to attach for 4 h, and then the medium was changed for all cells. Three dishes received medium alone and six dishes received medium containing 10^{-9} M MEL. Three dishes with MEL and three dishes without were placed in the control incubator; the other three dishes with MEL were placed in the MF exposure incubator. Each treatment was continuous for 7 days. On days 3 and 5, the medium was replaced with like medium, i.e., with or without MEL as required. Cells were then harvested and counted by hemocytometer in a manner blinded to treatment (see below).

Control experiments were performed using cells up to passage 10 from the original thaw. In one study, dishes of cells without MEL supplement, but otherwise prepared as described above, were placed in the control incubator and assayed periodically to determine the saturation density of the cells. All other tests were performed with cells that were below their saturation density on day 7; they reached a maximum of approximately 80–85 % confluent. In a second study, no differences in growth were observed in cells grown in either incubator when neither was energized to produce MF. A third study examined the influence of magnetic fields on cells without MEL. Five dishes of cells, prepared from a newly thawed sample, were plated on dishes without MEL supplement and placed in both control and field exposure incubators and treated continuously for 7 days. No influence of MF was observed on cell growth at day 7 for cells without MEL. Media changes and assay were performed as described above.

In the TMX study, cells were plated at a density of approximately 3.2×10^4 per 35 mm dish on 48 dishes, 24 with and 24 without 0.25×10^{-7} M TMX. Twelve dishes with TMX and 12 dishes without were placed in each of two exposure chambers. One exposure chamber was energized with MF and the other chamber was unenergized. Treatment was continuous, without any change of medium until dishes were removed for assay; cells were approximately 70–85% confluent on day 7. Assays were performed blinded to treatment condition. Five replicate experiments were conducted with one incubator energized, and four replicate experiments were conducted with the other incubator energized. We used cells up to 12 passages from the original thaw for these tests.

Assay of Cell Number

In the MEL study, the number of cells per dish on day 7 was determined by hemocytometer counting of the samples, using sample labels that did not indicate treatment condition (i.e., the experimental protocol blinded treatment conditions until after counting).

In the TMX study, the treatment condition was also blinded during the assay. For the TMX tests, the numbers of cells per dish at days 4, 5, 6, and 7 were determined using a detergent treatment (Dr. Thomas Wiese, personal communication) to lyse the plasma membrane of the cells and provide nuclei that were then enumerated in a Coulter Counter (Coulter Corp, model Z1, Miami, FL). In this procedure, the cells on each dish were rinsed with 2 ml of 0.15 M NaCl, then incubated at room temperature for 10 min in a 1 ml hypotonic solution (1.5 mM $MgCl_2$ and 0.01 M HEPES) to swell the plasma membrane, followed by addition of 0.1 ml of 0.13 M Bretol in 3% v/v glacial acetic acid. Phase contrast microscopy demonstrated that the cells contained single nuclei.

Statistical Analysis

A two factor (replication and treatment) analysis of variance was used to examine the difference among the control, MEL, and MEL plus MF treatments. The Ryan-Einot-Gabriel-Welsch (REGWF) multiple comparison procedure [Ryan, 1960; Einot and Gabriel, 1975; Welsch, 1977] was then used to determine which treatments were different from each other. The results of field exposure of cells without MEL were analyzed by one way analysis of variance.

In the TMX study, three dishes of each treatment were removed from each incubator and assayed on days 4, 5, 6, and 7 of treatment. On a given day, three counts from each dish were averaged. Next, the average of the three dishes at each treatment condition was calculated. This resulted in two counts in each incubator for each day and replication combination. A ratio was calculated for each incubator by dividing the averaged count with TMX present to the averaged count without TMX (multiplied by 100 to express the value as percentage). An analysis of variance for incomplete blocks was done on the ratios of TMX treated to TMX free cells from each incubator to test for MF, incubator, and MF-by-incubator effects for each day.

RESULTS

Controls

The cells were still in their logarithmic growth range on day 7 in both the MEL and the TMX studies (see Figure 1, which is from the TMX study), indicating that cell growth conditions were not limiting. Further, MF alone (i.e., on cells without MEL or TMX) had no effect on MCF-7 cell proliferation (Table 1, from the MEL study; Figure 2, from the TMX study). These results are in agreement with the report of Liburdy et al. and confirm that the control and exposure incubators were matched for cell growth conditions.

MEL Study

Pooled cell number results for three replicate tests of MEL and MEL plus MF treatments were compared to controls. The two factor (replication and treatment) analysis of variance indicated significant

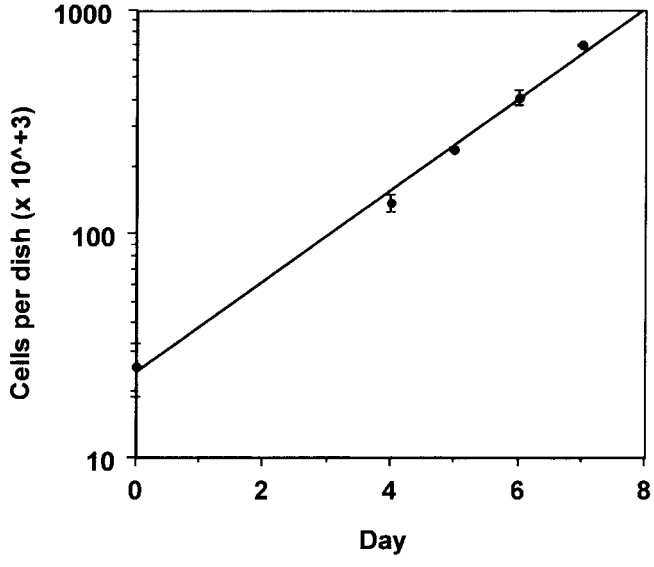

Fig. 1. Growth Phase of Cells in the TMX study. Cells are in logarithmic phase of growth through day 7. Data is a typical example (mean, SD, n = 3) for all trials.

TABLE 1. Magnetic Fields Do Not Affect the Growth Rate of MCF-7 Cells When the Medium is Not Supplemented With Melatonin

	Control	Exposed
Magnetic field	$< 0.2\,\mu T_{rms}$	$1.2\,\mu T_{rms}$
Mean cells/dish ($\times 10^6$; SE)[a]	2.79 (0.04)	2.76 (0.05)
n	5	5

[a]A one-way analysis of variance demonstrated that the mean number of cells/dish in the two treatment conditions are not statistically different, $P = 0.713$.

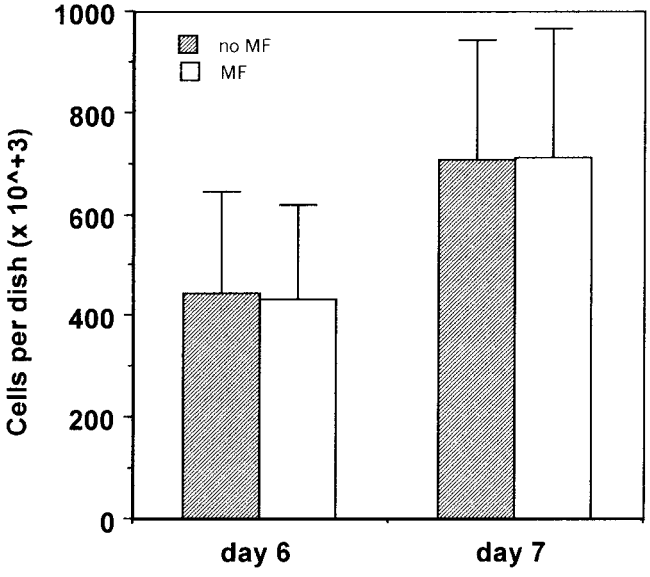

Fig. 2. There is no effect of MF on TMX-free cell growth. The growth of TMX-free cells in energized and not energized incubators (n = 9). Therefore, the MF treatment does not bias the analysis.

TABLE 2. Influence of Melatonin and of Melatonin and Magnetic Fields on Cell Proliferation

	Control	MEL	MEL & MF
B field	$< 0.2\,\mu T_{rms}$	$< 0.2\,\mu T_{rms}$	$1.2\,\mu T_{rms}$
Mean cells/dish[a]	1.38	1.15	1.39
SE	0.15	0.14	0.14
n	9	9	9

[a]($\times 10^6$).

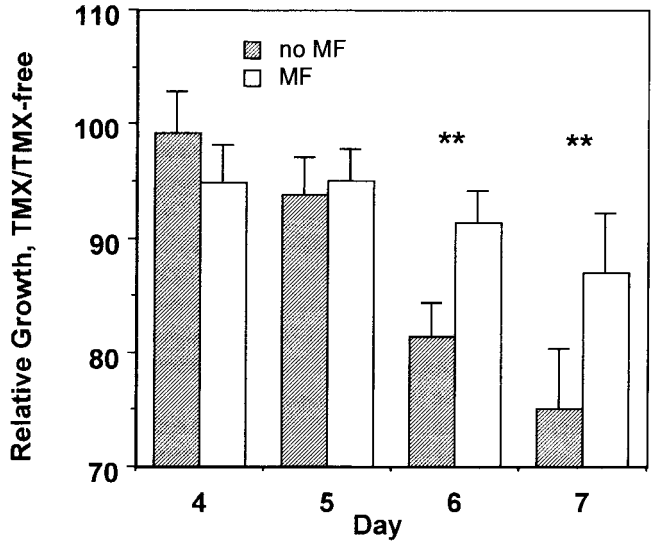

Fig. 3. Cell growth (TMX-treatment/TMX-free) under MF and no MF conditions for days 4-7. The data in the figure are pooled from experiments in which cultures were exposed in one of the two incubators and the other contained the unexposed controls (n = 5) and from experiments in which the roles of the incubators were reversed (n = 4; total n = 9). Asterisks indicate statistically significant differences ($P < 0.001$) on days 6 and 7.

($P < 0.001$) differences among the three treatments. The REGWF multiple comparison procedure was used to determine which treatments were different from each other. The results showed that the control and MEL & MF treatment means were not significantly different, but both means were significantly larger (16.7%; $P < 0.001$) than the MEL mean (Table 2).

Tamoxifen Study

An analysis of variance for incomplete blocks was done on the ratios of TMX treated to TMX free cells from each incubator to test for MF, incubator, and MF-by-incubator effects for each day. The analysis showed a statistically significant effect ($P < 0.001$) for MF on days 6 and 7 and no effect ($P > 0.05$) on any of the days due to incubator and MF-by-incubator interactions. The overall results are given in Figure 3, which shows the relative growth of cells (TMX treatment/TMX free) under MF and no MF conditions for days 4–7. The data in the figure pools results from experiments in which cultures were exposed in one of the two incubators and the other contained the unexposed controls (in one case, n = 5) and data in which the roles of the incubators were reversed (in this case, n = 4; total n = 9). Asterisks indicate statistically significant differences ($P < 0.001$) with respect to the effect of MF on days 6 and 7. These results agree with the findings of Harland and Liburdy [1997].

Figure 4 shows the excess growth of TMX treated cells compared to TMX free cells in the presence of MF. Results from day 6 and 7 are shown for each energized incubator (incubator #1, n = 4; incubator #2, n = 5). The results show that cell growth, i.e., the partial removal by MF of TMX induced growth inhibition, is the same regardless of which incubator was energized.

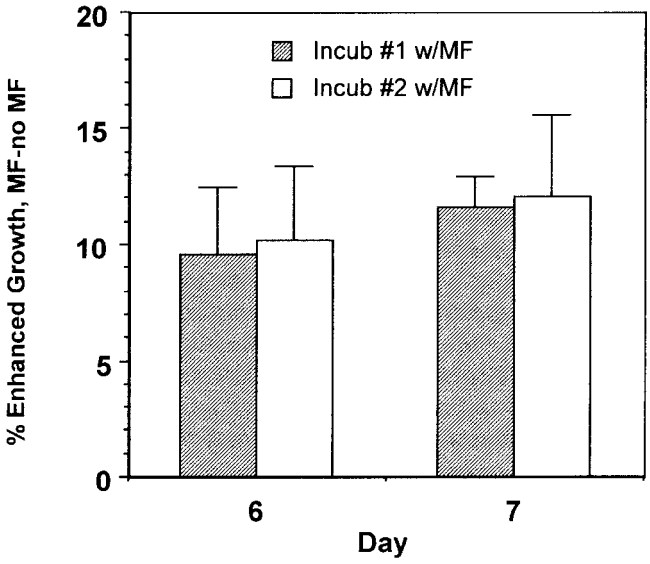

Fig. 4. The excess growth of TMX-treated cells compared to TMX-free cells in the presence of MF. Results from day 6 and 7 are shown for each energized incubator (incubator #1, n = 4; incubator #2, n = 5). MF effect is the same regardless of which incubator was energized.

DISCUSSION

This report describes the results of two studies, one a limited and the other a more expansive experimental design. The MEL study was undertaken to test only the effect of MF on day 7 reported by Liburdy et al. [1993a]. The experimental design to meet this objective was optimized to economize investigator time. The results in this study, a 16.7% reduction in cell number in MEL treated cells compared to controls on day 7 of treatment, are consistent with the results reported by Liburdy et al., which showed an 18% reduction in cell growth [Liburdy et al., 1993a; Figure 4a]. There is further agreement in the results of the MF treatment; there was no difference in cell number between control and MEL treated cells that were also exposed to magnetic fields.

The TMX study was designed to use a more efficient cell counting method. Thus, the design incorporated the protocol of Harland and Liburdy [1997] and was further expanded to examine ancillary issues of interest. The results in our study were consistent with those reported by Harland and Liburdy [1997]. They reported 33 and 40% inhibition of MCF-7 cell growth by TMX in control conditions (i.e., without MF) on days 6 and 7, respectively. They also reported a nil and 18% inhibition, respectively, in similar chemical conditions but with a MF present [Harland and Liburdy, 1997; Figure 3]. The extent of growth inhibition in our study was less, 18.6 and 25% inhibition in control (no MF) conditions on days 6 and 7, respectively, and 8.7 and 13.1% inhibition under similar chemical treatment with MF exposure. We believe the discrepancy may be due to the reduced concentration of TMX we used: 0.25×10^{-7} M vs. 1×10^{-7} M in Harland and Liburdy's 1997 study.

In both the MEL and the TMX studies, there were deviations from the Liburdy lab protocol: a) In our study the growth of stock MCF-7 cells was in an ordinary incubator without magnetic shielding; nevertheless the ambient 60 Hz fields were less than $0.2\,\mu T_{rms}$ as required by the protocol, b) we used Helmholtz rather than the Merritt coils used by the Liburdy laboratory to generate the MF, c) in our TMX study, the number of cells on each dish were enumerated using a Coulter counter rather than the hemocytometer used in Liburdy's laboratory. Despite these differences, the results obtained in our lab independently confirmed the basic experimental findings reported by Harland and Liburdy [1997]. A critical element of this replication effort was the constructive communication established and maintained with the original investigators.

The mechanism(s) by which 60 Hz magnetic fields influence the actions of MEL- and of TMX-induced inhibition of MCF-7 cell growth is not known. Could the cytotoxic effects of these chemicals, particularly TMX, be involved in the day 6 and day 7 sensitivity to magnetic fields? It is unlikely that the MEL induced effect is operating through a cytotoxic mechanism. The concentration of MEL used in this study was 10^5 times smaller than a reported cytotoxic level ($\sim$20 mM; Kojima et al., [1997]). Furthermore, cells were counted by hemocytometry using Trypan blue dye, which is used to indicate plasma membrane integrity, a measure of cell viability. No differences in staining were noted between different treatment populations. For the TMX studies, the concentration (2.5×10^{-8} M) was well below reported cytotoxic levels (greater than 10^{-6} M; Clarke et al., [1990]). In our TMX study, we noticed no or very few cells floating in the cultures on any day when they were removed from the incubators, so there was no obvious toxicity. Although cell toxicity was not specifically tested in the TMX study, we believe it is not involved in the magnetic field sensitivity that occurs on day 6 and day 7.

Why do cells treated with TMX, as well as cells treated with MEL [Liburdy et al., 1993b], only respond to fields after 6 or 7 days of growth? We do not know why this is true. It occurs over a range of final cell concentrations on day 7 while the cells are still in log phase of growth; nevertheless the MF sensitivity could be a cell density related phenomenon. Both MEL and

TMX have multiple and sometimes opposing actions in cells. MEL reduces [Vanecek and Vollrath, 1989; Godson and Reppert, 1997] and TMX increases [Lopes et al., 1990] cAMP levels in cells. MEL increases pKC via its action to increase inositol triphosphate in some cells [McArther et al., 1997] and yet decreases pKC via its action reducing DAG, AA, and $[Ca]_i$ in other cells [Vanecek and Vollrath, 1990]. TMX can inhibit pKC by directly binding to the ATP binding region [O'Brian et al., 1985, 1986, 1988; Horgan et al., 1986], but in some cases TMX can activate pKC [Issandou et al., 1990; Bignon et al., 1991]. MEL mediates $[Ca]_i$ via its action on both InsP3 receptors in the endoplasmic reticulum [Zemkova and Vanecek, 1997] and on the Ca pump in the plasma membrane [Vanecek and Klein, 1992a,b; Vanecek 1995]; TMX reacts with calmodulin to inhibit cAMP phosphodiesterase, which increases cAMP [Lopes et al., 1990; Rowlands et al., 1995]. Receptors may be involved in the effects we observed because MEL acts on the G_i/G_0 superfamily of G proteins [Morgan et al., 1990], whereas TMX acts on the estrogen receptor [Coezy et al., 1982; Ecker and Katzenellenbogen, 1983] and also on calmodulin [Lopes et al., 1990; Rowlands et al., 1995]. Less specific actions may also be involved, because MEL increases membrane fluidity [Daniels et al., 1996; Garcia et al., 1997, 2000], whereas TMX decreases it [Clarke et al., 1990]. Thus it is possible that magnetic fields act on common processes that are perturbed in an opposite manner by these two chemicals. Future studies are needed to narrow these many possibilities.

This confirmatory study, along with similar confirmatory experiments reported to date only in abstracts by Luben et al. [1996], Luben and Morgan [1998], and Morris et al. [1998], demonstrate that continuous exposure to 1.2 μT_{rms}, sinusoidal, 60 Hz magnetic fields can influence chemically induced proliferation control processes in breast cancer cells in vitro. Further research is necessary to develop ''testable'' hypotheses to establish the underlying physical and biochemical mechanisms of action responsible for the observations so that the physiological relevance can be established. Reports by Molis et al. [1995] and MacGregor and Jordan [1998] suggest particular molecular pathways that should be studied in this context.

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