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Antagonism of Endogenous Growth Hormone–Releasing Hormone (GHRH) Leads to Reduced Proliferation and Apoptosis in MDA231 Breast Cancer Cells

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GHRH, in addition to stimulating the release of growth hormone (GH) from the pituitary, is a trophic factor for pituitary somatotrophs. Growth hormone–releasing hormone is also expressed in the gonads, gastrointestinal tract, pancreas, thymus, and lymphocytes, as well as in tumors of the pancreas, lung, central nervous system, and breast. Since GHRH has mitogenic effects, we examined the hypothesis that GHRH is an autocrine/paracrine growth factor in neoplastic breast tissue. The effect of disrupting endogenous GHRH on cell growth and apoptosis of MDA231 cells was examined through the use of a competitive GHRH antagonist, [*N*-acetyl-Tyr1, D-Arg2] fragment 1–29Amide (GHRHa). Cell proliferation was determined by direct cell counting and tritiated thymidine incorporation. Apoptosis was analyzed by examination of DNA laddering and nuclear condensation. GHRHa resulted in a dose-dependent, transient, and reversible decrease in cell number, proliferation rate, and tritiated thymidine uptake. Conversely, GHRHa led to a marked and dose-dependent increase in both DNA laddering and nuclear condensation. These results indicate that disruption of endogenous GHRH action in MDA231 cells results in both decreased cellular proliferation and increased apoptosis. Taken together, the findings suggest that endogenous GHRH acts as an autocrine/paracrine factor in the regulation of growth of at least some breast cancer cell types.

Key Words: Growth hormone–releasing hormone; neoplasia, breast; growth factor; apoptosis; autocrine/paracrine growth control.

Introduction

The hypothalamic neuropeptide growth hormone–releasing hormone (GHRH) stimulates growth hormone (GH) synthesis and secretion from the pituitary and is a critical trophic factor promoting development and proliferation of pituitary somatotrophs *(1–8)*. GHRH is also expressed in a limited set of other tissues, including lymphocytes, placenta *(9,10)*, gut *(11)*, kidney *(12)*, thymus *(13)*, and testis *(10,14)*, where it is assumed to play an autocrine/paracrine role. However, the physiology of extrahypothalamic GHRH has not been well studied, although mitogenic activity has been reported in lymphocytes and testicular germ cells *(15–17)*, suggesting that a trophic role for GHRH may not be unique to the pituitary somatotroph. Recently, we *(18)* and others *(19)* have demonstrated that GHRH activates the mitogen-activated protein kinase (MAPK) pathway, as well as cellular proliferation in somatotroph cell lines, providing a potential signaling framework for mitogenic actions.

GHRH is also known to be expressed in tumors of the central nervous system, lungs, and gastrointestinal (GI) tract *(20)*. Indeed, GHRH was originally isolated from pancreatic tumors, and ectopic secretion of GHRH is a well-described cause of acromegaly. More recently, expression has been demonstrated in tumors of the breast *(21)*, prostate *(22)*, ovary, and endometrium *(23–25)*. Furthermore, GHRH receptor antagonists (GHRHa) have been reported to have antitumorigenic activity in a variety of transformed human cell lines, including GI tract, renal, prostate, ovarian, and breast *(26–31)*. However, the mechanism of action of these antagonists to inhibit tumor growth, as well as the underlying role of GHRH itself in these tumors, remains unclear.

To study GHRH biology in extrahypothalamic tumors, we were interested in establishing an in vitro model amenable to molecular dissection. Since breast cancer cell lines express endogenous GHRH *(21,32)*, we first demonstrated that these cell lines also express the GHRH receptor and then examined the effect of disrupting the autocrine/paracrine actions of GHRH on proliferation and apoptosis of these cells using a GHRH receptor antagonist.

Results

As shown in Fig. 1, reverse transcriptase polymerase chain reaction (RT-PCR) of total RNA from MDA231 cells using hGHRHr-specific primers detected the presence of a 953-bp transcript identical in size to that present in human

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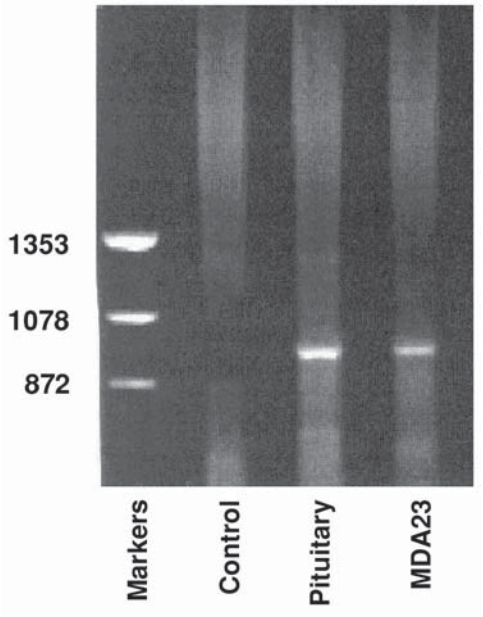

Fig. 1. RT-PCR amplification of total RNA from a human pituitary tumor and MDA231 cells. RT-PCR amplification (35 cycles) was performed using 1 μg of total RNA prepared from MDA231 cells and a human pituitary tumor and primers designed to amplify a 953-bp segment of the hGHRH receptor as described in Materials and Methods. Cloning and direct sequencing confirmed the identity of the transcript as hGHRH receptor mRNA.

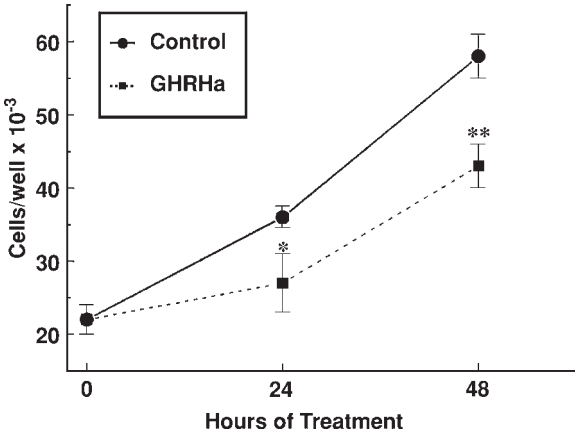

Fig. 2. Effect of GHRHa on MDA231 cell counts in vitro. MDA231 cells were plated at 8000/cm in 96-well plates and allowed to attach overnight in Dulbecco's modified Eagle's medium (DMEM)/2% fetal calf serum (FCS). The medium was replaced with DMEM without serum, and the competitive GHRH antagonist GHRHa was applied for 1 h as described in Materials and Methods. The medium was then brought to 2% FCS and allowed to incubate until the indicated times. In all experiments, control cells were treated with the same final concentration of vehicle alone. At the time of counting, the medium was aspirated, and the cells were removed following treatment with 50 μL of trypsin and resuspended in 150 μL of PBS. Four 0.1-μL samples of each well were counted by hemocytometer, with eight replicates per treatment. Values represent the mean ± SEM; $n = 8$ replicates at each time point for each treatment. *$p < 0.05$; **$p < 0.01$.

pituitary tissue. No transcript was present in amplifications lacking input RNA (control lane). Cloning and direct sequencing confirmed the identity of the transcript as full-length hGHRH receptor mRNA. Western blot analysis confirmed previous reports of immunoreactive GHRH in extracts from MDA231 cells *(32)*.

The effect on cell growth of disruption of endogenous GHRH signaling was examined using the hGHRH antagonist, [*N*-acetyl-Tyr1, D-Arg2] fragment 1-29Amide (Sigma, St. Louis, MO). As shown in a representative experiment (Fig. 2), a single treatment of MDA231 cells with 3 μ*M* antagonist resulted in an approx 25% decrease in cell number after 24 h. Subsequently, cell numbers increased in parallel with control cells, indicating that the effect is transient and reversible. A second treatment after 24 h led to an additional 24 h of inhibition of the increase in cell number. This inhibition by GHRHa was also dose dependent. As shown in a representative experiment (Fig. 3), exposure for 24 h to GHRHa resulted in decreases in cell number ranging from 6% at 40 n*M* to 25% at 5 μ*M*. Higher doses did not lead to decreases >25%.

To determine the mechanism responsible for the reduction in cell number caused by exposure to GHRHa, the effect of GHRHa on measures of cellular proliferation and mitosis was examined. As shown in a representative experiment (Fig. 4), exposure of MDA231 cells to a single dose of 3 μ*M* GHRHa resulted in a rapid and transient decline in thymidine uptake followed by uptake parallel to control cells. This change in thymidine uptake indicates a decrease in DNA synthesis and suggests a decrease in cellular proliferation. When cells were exposed to a second dose of GHRHa after 4 h, tritiated thymidine uptake was inhibited for an additional 4 h followed by recovery and uptake parallel to control cells.

To evaluate whether antagonism of endogenous GHRH also decreased cell counts through promotion of apoptosis, the effect of GHRHa on apoptosis was determined using two independent techniques. As shown in Fig. 5, exposure of MDA231 cells to 3 μ*M* GHRHa led to a marked increase in DNA laddering compared to vehicle alone. In addition, exposure of MDA231 cells to 3 μ*M* GHRHa for 24 h increased the frequency of appearance of condensed nuclei after staining with Hoechst dye. To quantify this increase, the number of condensed nuclei present in a ×100 field (four fields per slide, eight slides per treatment) was counted. As shown in Fig. 6, after 24 h in the presence of 3 μ*M* GHRHa, the frequency of apoptotic cells increased by 60–75% in independent experiments. However, even after the increase in apoptotic frequency following GHRHa treatment, the overall rate of apoptosis remained limited, with approx 5% of cells in

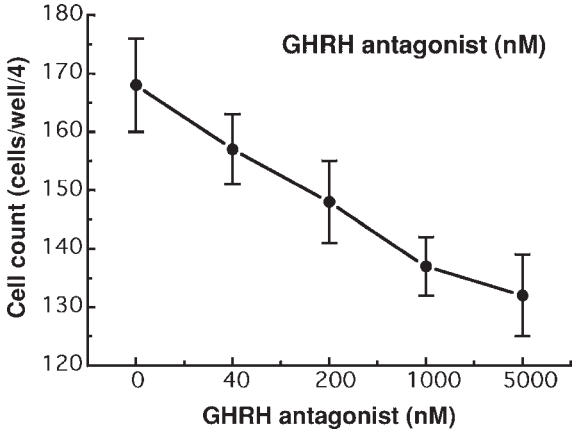

Fig. 3. Dose-dependent effect of GHRHa on MDA231 cell counts in vitro. MDA231 cells were plated at 8000/cm in 96-well plates and allowed to attach overnight in DMEM/2% FCS. The medium was replaced with DMEM without serum, and the competitive GHRH antagonist GHRHa was applied for 1 h as described in Materials and Methods. The medium was then brought to 2% FCS and allowed to incubate until the indicated times. In all experiments, control cells were treated with the same final concentration of vehicle alone. At the time of counting, the medium was aspirated, and the cells were removed following treatment with 50 µL of trypsin and resuspended in 150 µL of PBS. Four 0.1-µL samples of each well were counted by hemocytometer, with eight replicates per treatment. Values represent the mean ± SEM; n = 8 replicates at each time point for each treatment.

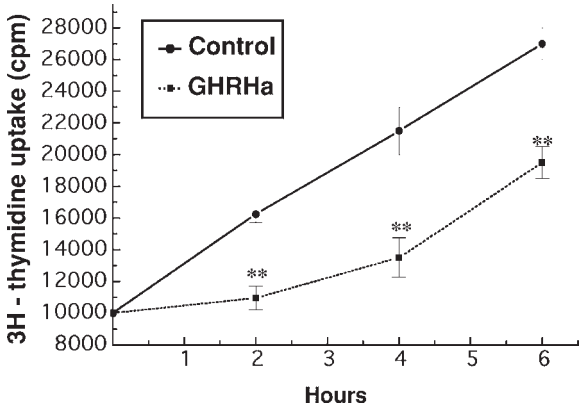

Fig. 4. Effect of GHRHa on ^{3}H-thymidine uptake by MDA231 cells in vitro. MDA231 cells were plated at 8000/cm in 96-well plates and allowed to attach overnight in DMEM/2% FCS. The medium was replaced with DMEM without serum and the competitive GHRH antagonist GHRHa was applied for 1 h as described in Materials and Methods. The medium was then brought to 2% FCS. Four hours after GHRHa treatment, 0.8 µL of ^{3}H-thymidine was added to each well. At the indicated times, cells were washed thrice with 150 µL of phosphate-buffered saline (PBS), followed by 25 µL of 10% trichloroacetic acid (TCA). After 5 min, 100 µL of 0.1 *M* NaOH was added followed by 27.5 µL of 0.*1M* HCl. The entire content of the well was transferred to scintillation counter tubes and counted for 10 min. Values represent the mean ± SEM; n = 8 replicates at each time point for each treatment. **$p < 0.01$.

a field of 2000 demonstrating signs of apoptosis at 24 h, a rate consistent with the decrease in cell numbers at 24 h.

Discussion

The data presented here indicate that antagonism of the endogenous GHRH autocrine/paracrine system in MDA231 breast cancer cells leads to inhibition of cell proliferation, as well as increased cellular apoptosis, the combination of which leads to decreased cell number. The effect of GHRH antagonist is dose-dependent, transient, and reversible. These results are consistent with previous demonstrations of an inhibitory effect of GHRH antagonists on a variety of reproductive and GI tract tumors, extending these observations to provide initial information regarding the mechanism of the effect of GHRH antagonists.

The decrease in cell numbers seen in the MDA231 cells exposed to GHRH antagonist is unlikely to be owing to nonspecific toxicity. The effects of the antagonist are transient and completely reversible, suggesting a physiologic rather than catastrophic event. Furthermore, the effect of the antagonist on cellular proliferation is relatively modest even at maximal doses, suggesting that the effect is limited to certain cells, perhaps in a particular physiologic state or position in the cell cycle, rather than a generalized toxic effect on all cells. Finally, the decrease in cell number is associated with DNA laddering and nuclear condensation, features characteristic of apoptosis rather than nonspecific cell death.

The implications of changes in thymidine uptake are arguable. On the one hand, a decrease in uptake may reflect decreased rates of DNA synthesis (i.e., reduced mitosis). Alternatively, decreased uptake may indicate reduced rates of DNA repair processes. However, in the current experiments, the association of decreased thymidine uptake with the subsequent reduction in cell number strongly suggests that exposure to GHRH antagonist is promoting a decrease in the rate of mitosis and cell proliferation. Furthermore, while it is conceivable that the decrease in thymidine uptake reflects the loss of DNA synthesis by cells undergoing apoptosis, the degree of reduced cell number appears to exceed what can be accounted for by apoptosis alone, implying that at least a portion of the cell number reduction is a consequence of reduced cellular proliferation.

The concept of GHRH as a promoter of cellular proliferation is not in itself novel. Within the hypothalamic pituitary axis, extensive evidence supports the role of GHRH in the development and proliferation of GH-secreting somatotrophs. GHRH stimulates the expression of both the GH gene and c-*fos*, and enhances somatotroph proliferation in

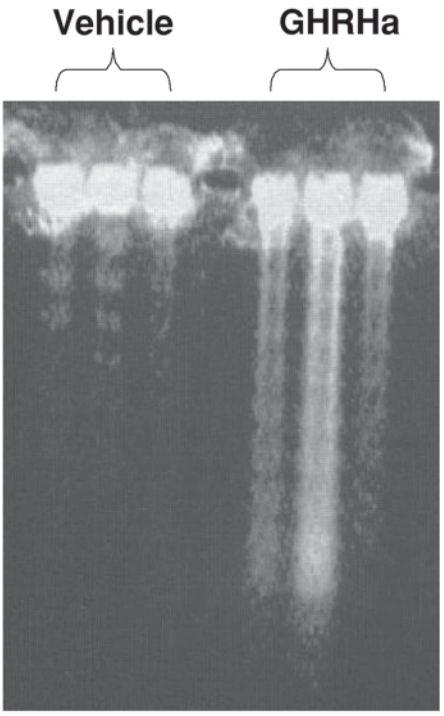

Fig. 5. Effect of GHRHa on DNA laddering in MDA231 cells. MDA231 cells were grown as described in 3.5-cm plates overnight. Following treatment with GHRHa as described, the volume of medium was increased to 3 mL. Cells were harvested 12 h after GHRHa treatment, pelleted by centrifugation, and washed once in PBS. Cells were lysed in 20 µL of lysis buffer (50 m*M* Tris, pH 8.0; 10 m*M* EDTA; 0.5% sodium dodecyl sulfate [SDS]; 0.5 mg/mL of proteinase K) and heated to 50ºC for 1 h. The mixture was then heated to 90ºC for 3 min to deactivate the proteinase K, treated with 10 µL of RNase A to a final concentration of 0.5 µg/mL in TE, and heated to 50ºC for 1 h. Samples were separated by electrophoresis through 2% agarose with ethidium bromide and visualized (Alphaimager). Each lane represents the total DNA sample obtained from a single replicate, with three replicates per treatment.

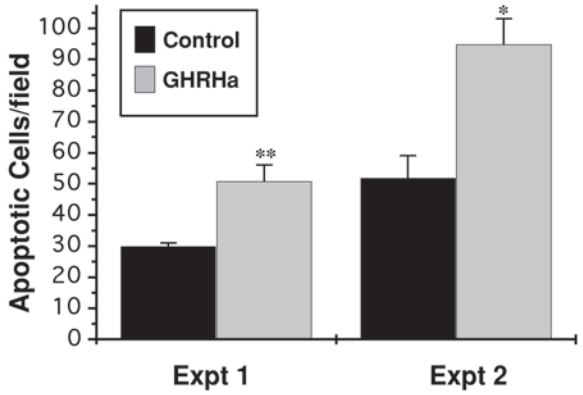

Fig. 6. Effect of GHRHa on nuclear condensation of MDA231 cells in vitro. Cells were grown as described in 100 µL of DMEM/2% FCS on chamber slides overnight. GHRHa was added in 80 µL of DMEM and allowed to incubate for 1 h. The medium was then brought to 2% FCS. Twenty-four hours after GHRHa treatment, cells were washed briefly with PBS, fixed in 4% paraformaldehyde for 10 min, followed by 70% EtOH in glycine buffer for 10 min at –20ºC. Cells were then washed in PBS, incubated with Hoechst dye (8 µg/mL) for 15 min at room temperature, and rinsed three times in PBS. Slides were then masked and cells visualized by fluorescent microscopy and apoptotic cells counted (four fields per slide, eight slides per treatment). Values represent the mean ± SEM in two independent experiments. *$p < 0.05$; **$p < 0.01$.

vitro *(4–6,18,33–35)*. Long-term exposure to GHRH in vivo results in somatotroph hyperplasia in animals *(36,37)* and humans *(38,39)*. Conversely, rats in which GHRH action is transiently impaired during the neonatal period have reduced pituitary size and somatotroph cell number *(7,40–42)*. Similarly, resistance to GHRH action, as in the *lit* mouse *(43, 44)* or *dw* rat *(2)*, or congenital absence of GHRH, as in the GSH-1 knockout mouse *(1)*, is associated with marked somatotroph hypoplasia.

However, the mechanism by which GHRH promotes cellular proliferation is unclear. In pituitary somatotrophs, analogs of cyclic adenosine monophosphate (cAMP) and somatotroph-targeted expression of cholera toxin in transgenic animals induce cellular proliferation in culture *(35,45)*. Conversely, GH-promoter driven overexpression of dominant negative CREB leads to somatotroph hypoplasia *(8)*, a finding interpreted to indicate that inhibition of the transcriptional effects of cAMP prevents the genomic and proliferative effects of GHRH. Recently, we *(18)* and others *(19)* have demonstrated that GHRH activates the MAPK pathway, as well as cellular proliferation in somatotroph cell lines. Furthermore, proliferation in response to GHRH was prevented by agents that prevent activation of MAPK, strongly implying that GHRH promotes proliferation, at least in part, through activation of the MAPK pathway.

The question of which receptor is transducing extrahypothalamic actions of GHRH has been somewhat controversial. In some cases, attempts at identifying the GHRH receptor in GHRH antagonist–responsive tumors has been unsuccessful *(21,29,46)*, and it has been suggested that GHRH may be acting through related vasoactive intestinal peptide or pituitary adenylate cyclase-activating peptide (PACAP) receptors *(47,48)*. However, in the case of MDA231 breast cancer cells, the presence of GHRH receptor mRNA suggests that the actions of GHRH on cellular proliferation are likely mediated by the GHRH receptor itself.

In summary, the results of these experiments indicate that exposure of MDA231 breast cancer cells to a GHRH receptor antagonist in vitro results in reduced cell numbers. Furthermore, the experiments provide evidence that the decrease in cell number reflects both decreased cellular proliferation, as indicated by decreased tritiated thymidine uptake, and increased cellular apoptosis, as indicated by increased DNA laddering and nuclear condensation. Taken together, the data suggest that disruption of endogenous GHRH receptor signaling results in disruption of normal MDA231 cellular dynamics, leading to decreased proliferation and survival

of the breast cancer cells. By extension, these results imply that endogenous GHRH supports MDA231 cell proliferation and inhibits apoptotic pathways.

Materials and Methods

Cell Culture

MDA231 cells, originally obtained from American Type Culture Collection, were grown to confluency under standard conditions in DMEM supplemented with 10% FCS. Prior to experiments, the cells were removed following treatment with PBS/2% EDTA; plated at 8000/cm in 96-, 24-, or 6-well plates; and allowed to attach overnight in DMEM/2% FCS. For treatments, the medium was replaced with DMEM without serum and treatments applied for 1 h, following which medium was brought to 2% FCS and maintained until harvest. The competitive GHRH antagonist GHRHa was dissolved in 2% acetic acid/1% insulin-free bovine serum albumin to a stock concentration of 1 m*M*. In all experiments, control cells were treated with the same final concentration of vehicle alone.

RT-PCR Amplification

Total RNA was prepared from MDA231 cells and a human pituitary tumor using commercial reagents. RT-PCR amplification (35 cycles) was performed using 1 μg of total RNA from each tissue and primers designed to amplify a 953-bp segment of the hGHRH receptor as previously described *(49)*.

Cell Counts

Cells were grown as described in 100 μL of DMEM/2% FCS in a 96-well plate overnight. GHRHa was added in 80 μL of DMEM and allowed to incubate for 1 h. The medium was then brought to 2% FCS and allowed to incubate until the indicated times. At the time of counting, the medium was aspirated, and the cells were removed following treatment with 50 μL of trypsin and resuspended in 150 μL of PBS. Four 0.1-μL samples of each well were counted by hemocytometer, with eight replicates per treatment.

Tritiated Thymidine Uptake

Cells were grown as described in 100 μL of DMEM/2% FCS in a 96-well plate overnight. GHRHa was added in 80 μL of DMEM and allowed to incubate for 1 h. The medium was then brought to 2% FCS. Four hours after GHRHa treatment, 0.8 μL of ^{3}H-thymidine was added to each well. At the indicated times, cells were washed thrice with 150 μL of PBS, followed by 25 μL of 10% TCA. After 5 min, 100 μL of 0.1 *M* NaOH was added followed by 27.5 μL of 0.*1M* HCl. The entire content of the well was transferred to scintillation counter tubes and counted for 10 min. Each treatment was examined in eight replicates.

DNA Ladder

Cells were grown as described in 3.5-cm plates overnight. Following treatment with GHRHa as described, the volume of medium was increased to 3 mL. Cells were harvested 12 h after GHRHa treatment, pelleted by centrifugation, and washed once in PBS. Cells were lysed in 20 μL of lysis buffer (50 m*M* Tris, pH 8.0; 10 m*M* EDTA; 0.5% SDS: 0.5 mg/mL of proteinase K) and heated to 50ºC for 1 h. The mixture was then heated to 90ºC for 3 min to deactivate the proteinase K, treated with 10 μL of RNase A to a final concentration of 0.5 mg/mL in TE, and heated to 50ºC for 1 h. Samples were separated by electrophoresis through 2% agarose with ethidium bromide and visualized (Alphaimager).

Hoechst Staining

Cells were grown as described in 100 μL of DMEM/2% FCS on chamber slides overnight. GHRHa was added in 80 μL of DMEM and allowed to incubate for 1 h. The medium was then brought to 2% FCS. Twenty-four hours after GHRHa treatment, cells were washed briefly with PBS, fixed in 4% paraformaldehyde for 10 min, followed by 70% EtOH in glycine buffer for 10 min at –20ºC. Cells were then washed in PBS, incubated with Hoechst dye (8 μg/mL) for 15 min at room temperature, and rinsed three times in PBS. Slides were masked so that the reader would be unaware of the treatment exposure, cells were visualized by fluorescent microscopy, and apoptotic cells were counted (four fields per slide, eight slides per treatment).

Statistical Analysis

Where indicated, data were analyzed by one-way analysis of variance followed by post-hoc analysis with the Neuman-Keuls test.

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