

# Stabilization of Mammary-derived Growth Inhibitor Messenger RNA by Antiestrogens<sup>1</sup>

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## ABSTRACT

Mammary-derived growth inhibitor (*MDGI*) is a tumor suppressor gene that is maximally expressed in terminally differentiated mammary epithelial cells. The *MDGI* gene is silenced in human breast cancer cell lines and in many primary human and experimental breast tumors. We demonstrate that the antiestrogens 4-hydroxytamoxifen (4-OH tamoxifen) and ICI 182780 (ICI) stimulate *MDGI* expression *in vitro*. Dose-dependent *MDGI* mRNA accumulation was observed when ICI was added to the culture medium of mammary explants. Both 4-OH tamoxifen and ICI stabilized *MDGI* mRNA without affecting the transcription rate of the *MDGI* gene. Under estrogen-free conditions, the half-life of *MDGI* mRNA in control explants was approximately 6 h. This half-life was increased to 7.5 h in the presence of  $10^{-7}$  M 4-OH tamoxifen and to greater than 12 h in the presence of  $10^{-7}$  M ICI. There was a positive correlation between the antiproliferative activity of antiestrogens and their ability to stabilize *MDGI* mRNA. The up-regulation of expression of the *MDGI* tumor suppressor gene in normal breast tissue by antiestrogens may contribute to the protective activity of these compounds that is seen in mammary gland carcinogenesis experimental systems and to the decreased risk of contralateral cancer that is seen in women receiving tamoxifen therapy.

## INTRODUCTION

Antiestrogens are commonly used in the treatment of breast cancer and are currently being studied as compounds for breast cancer prevention (1–3). The pure antiestrogen ICI<sup>3</sup> (4) has activity in some tumors that are resistant to tamoxifen (5). ICI

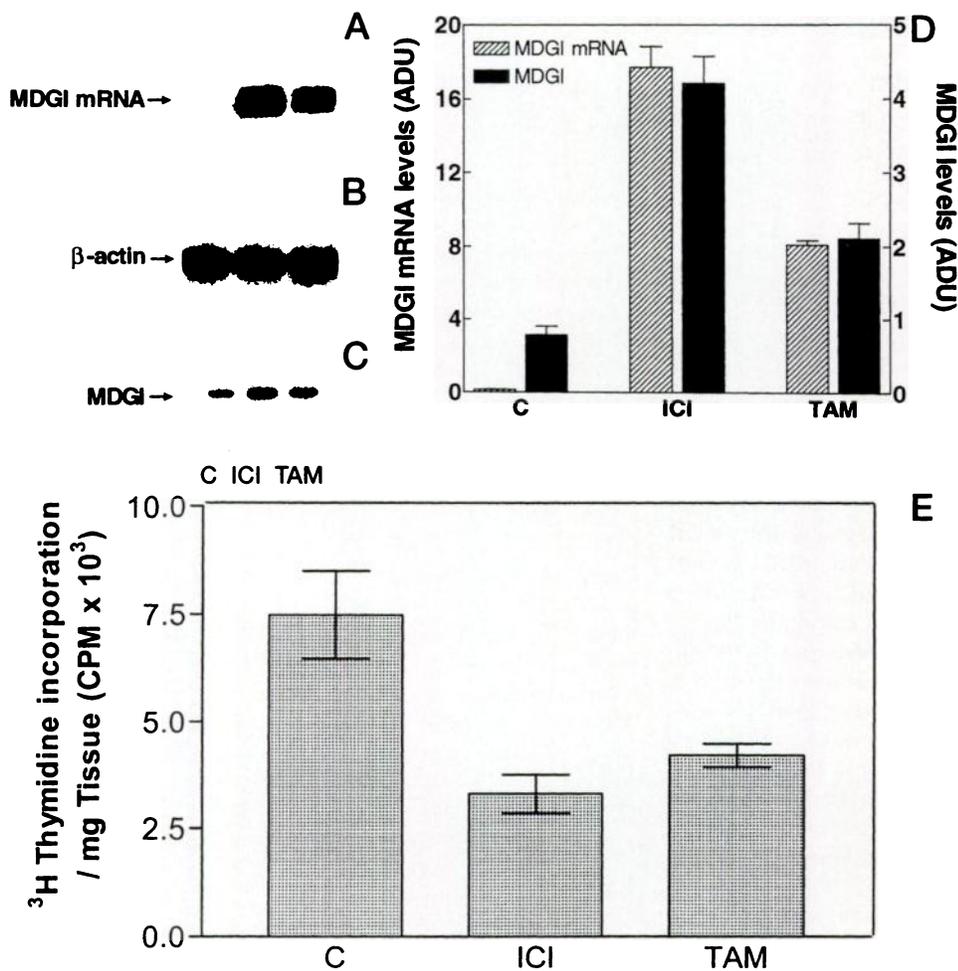
acts as a growth inhibitor in the complete absence of estrogen stimuli (6, 7) and actively regulates gene expression in a direction opposite to that of estrogens (7). We and others (7–9) have shown that ICI may have more potent antineoplastic activity than tamoxifen and that it is also associated with uterine involution rather than with uterine hypertrophy.

MDGI (also known as heart fatty acid-binding protein) is a member of the fatty acid-binding protein family of lipophilic intracellular proteins, which include retinoic acid-binding proteins (10–13). Immunocytochemical studies showed that MDGI was preferentially expressed by terminally differentiated mammary epithelial cells (14). A strong relationship also exists between onset of differentiation, inhibition of proliferation, and onset of MDGI expression (14). MDGI has certain properties that are compatible with regulatory or signal transduction functions. These include the presence of an Asn-Phe-Asp-Asp-Tyr consensus site for phosphorylation by tyrosine kinases (15), a differentiation-promoting effect on BLC6 murine pluripotent stem cells (16) and on mouse mammary epithelial cells (17), an inhibitory effect on yeast (*Saccharomyces cerevisiae*) proliferation (18), and inhibition of proliferation of Ehrlich mammary ascites cells (19). In addition, recent studies demonstrated that expression of a cDNA-encoding bovine MDGI reverted the transformed phenotype of MCF-7 breast cancer cells, further suggesting that the *MDGI* gene has tumor suppressor function (20). Epimutation was responsible for silencing this gene in a significant number of human breast cancers and human breast cancer cell lines (21).

## MATERIALS AND METHODS

**Mammary Explants.** Explants were prepared from the mammary gland of 14-day pregnant Sprague Dawley rats. This work was carried out under estrogen phenol red-free conditions. The gland was cleared of adhering tissues and then finely diced (to approximately 1-mm<sup>3</sup> cubes) with a surgical blade in a small amount of DMEM (Life Technologies, Inc.). Aliquots of the tissue (80–100 mg each) were cultured in phenol red-free DMEM supplemented with 5% double-charcoal-stripped serum, 5 µg/ml transferrin (Collaborative Research), and 1 µg/ml fibronectin (Collaborative Research; basal medium) containing various concentrations of ICI (4) or 4-OH tamoxifen (Sigma Chemical Co.; from  $10^{-9}$  M to  $10^{-7}$  M) for 48 h.

**Thymidine Incorporation.** Mammary explants were cultured and treated as described above for 48 h. Three aliquots of the tissue (80–100 mg each) were added to 1.35 ml of DMEM and incubated for 2 h in a shaking water bath at 37°C in the presence of 1 µCi/ml [<sup>3</sup>H]thymidine (ICN, Irvine, CA). The incubation was stopped by the addition of unlabeled thymidine solution. The medium was then quickly aspirated, and the tissue was washed twice with perchloric



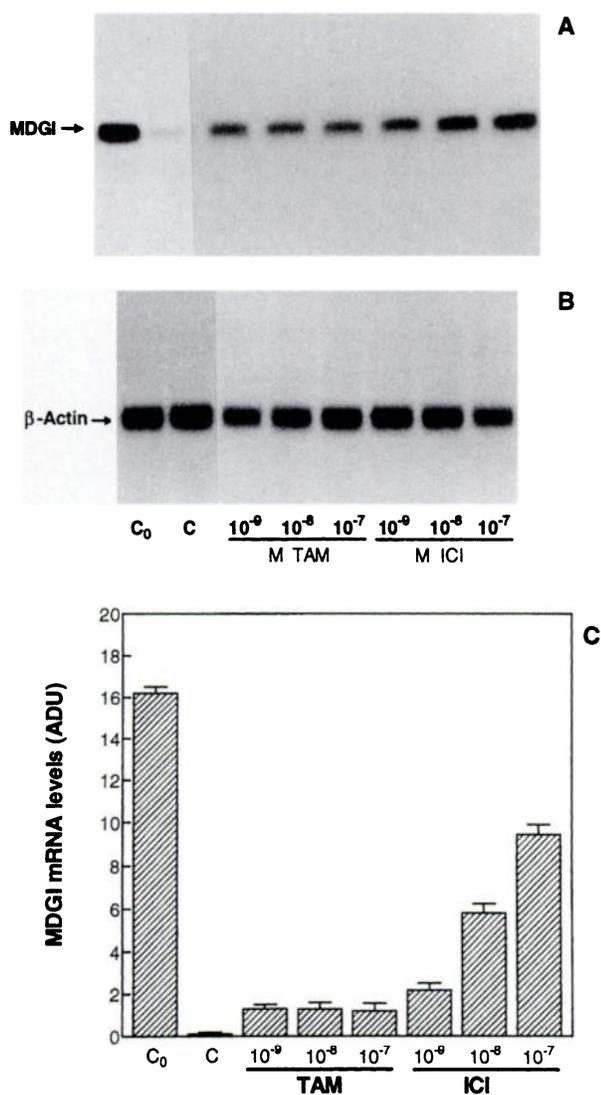
**Fig. 1** Effects of 4-OH tamoxifen and ICI on mammary explant DNA synthesis, MDGI mRNA levels, and MDGI accumulation. A–C, mammary explants were cultured in either SFPF medium (Lane C) or SFPF medium plus  $10^{-7}$  M 4-OH tamoxifen (Lane TAM) or  $10^{-7}$  M ICI (Lane ICI) for 48 h. Northern blots were performed as described in “Materials and Methods.” Western blotting of tissue extract (from equal protein) was performed as described in “Materials and Methods.” A, representative Northern blot for MDGI. B, representative Northern blot for  $\beta$ -actin. C, Western blots for MDGI. D, densitometric quantification. E, [ $^3$ H]thymidine incorporation. For ICI and 4-OH tamoxifen treatments, all groups yielded MDGI mRNA abundance, MDGI, and thymidine incorporation significantly different from the control ( $P < 0.05$ ; Mann-Whitney  $U$  test). Columns, means of quadruplicate experiments; bars, SE.

acid (5 g/100 ml) at 4°C. In preliminary studies, this procedure was found to remove all unincorporated [ $^3$ H]thymidine. The tissue was then blotted, weighed, and digested in 0.3 ml of Soluene-350 (Packard Instrument Co., Downers Grove, IL). Ten ml of scintillation fluid were added, and thymidine incorporation was determined. After correction for quenching, results were expressed as cpm per mg of tissue. The mean of three determinations was taken.

**Northern Blot Analysis.** Total RNA from cells was isolated as described (22) using RNeasy Lysis Buffer (Qiagen, Crawfordsville, IN). Northern blot analysis was performed as described (22). The blots were hybridized with a 680-bp mouse *MDGI* insert (17) as described previously (22), using  $1 \times 10^7$  cpm of the probe prepared by random primer synthesis (Pharmacia). Integrity and equal loading of RNA were verified by hybridizing the blots to a human  $\beta$ -actin insert (American Type Culture Collection). Quantitative analysis of gene expression was accomplished by densitometric scanning of autoradiographs.

**Nuclear Run-off Assay.** Explants were grown in phenol red-free medium supplemented with 5% double-charcoal-stripped fetal bovine serum for 24 h. Cells were washed with SFPF medium 3 times. Explants were treated with either

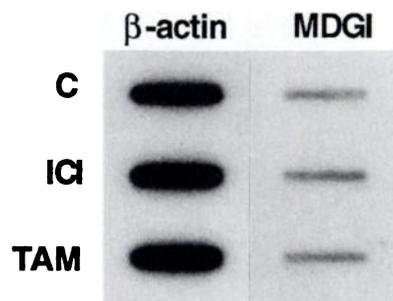
$10^{-7}$  M 4-OH tamoxifen or  $10^{-7}$  M ICI for 48 h in PFSF medium. Explants were blotted, suspended in ice-cold homogenization buffer [50 mM Tris (pH 7.5), 20 mM KCl, 5 mM  $MgCl_2$ , 0.5 M sucrose, 0.15 mM spermine, and 0.5 mM spermidine], and homogenized with 10 strokes at medium speed using a motor-driven Teflon-glass homogenizer. The homogenate was layered onto a 0.88 M sucrose cushion in ice-cold homogenization buffer and centrifuged for 5 min at 5000 rpm in an SS-34 rotor (Sorvall). The homogenate was homogenized once more as described above. The final nuclei pellets were suspended in buffer containing 10 mM Tris-HCl (pH 7.4), 3 mM  $CaCl_2$ , and 2 mM  $MgCl_2$ , repelleted, resuspended in glycerol storage buffer [50 mM Tris-HCl (pH 8.3), 40% glycerol, 5 mM  $MgCl_2$ , and 0.1 mM EDTA], and stored in liquid nitrogen until assay. Two hundred  $\mu$ l of frozen nuclei were added to 200  $\mu$ l of reaction buffer containing 10 mM Tris-HCl (pH 8), 5 mM  $MgCl_2$ , 0.3 M KCl, 1 mM ATP, 1 mM CTP, and 1 mM GTP, plus 0.5 mCi of [ $\alpha$ - $^{32}$ P]UTP (3000 Ci/mmol; ICN). After 30 min, the nuclei were digested with DNase I as described (23). Labeled RNA was obtained by phenol extraction followed by ethanol precipitation. Blots were hybridized with  $10^7$  cpm of labeled RNA for 48 h. Binding of mouse *MDGI* and  $\beta$ -actin cDNAs to nitrocellu-



**Fig. 2** Effects of 4-OH tamoxifen and ICI on MDGI mRNA abundance in mammary explants. Blots were performed using pooled total RNA from mammary tissue prior to culture ( $C_0$ ), using explants in basal medium (C), and using indicated doses of 4-OH tamoxifen (TAM) and ICI (ICI). Total RNA was extracted, and Northern blot analysis was performed as described in "Materials and Methods," using the mouse MDGI cDNA probe (A) and a  $\beta$ -actin probe as a loading control (B). C, densitometric scanning of MDGI mRNA levels after correction for equal loading using  $\beta$ -actin mRNA. For ICI- and 4-OH tamoxifen-treated groups, all yielded MDGI mRNA abundance significantly different from control ( $P < 0.05$ ; Mann-Whitney  $U$  test). Columns, means of quadruplicate experiments; bars, SE.

lose membrane, hybridization of labeled RNA to DNA immobilized on strips of nitrocellulose, and washings were performed as described (23).

**mRNA Stability.** Changes in MDGI mRNA stability that were induced by antiestrogens were carried out by the exposure of explants to  $10^{-7}$  M 4-OH tamoxifen,  $10^{-7}$  M ICI, or vehicle alone for 48 h. The transcription of cellular mRNA was inhibited by treating explants in parallel with 25  $\mu$ g/ml



**Fig. 3** Effects of 4-OH tamoxifen and ICI on MDGI gene transcription. Explants were treated with either SFPP medium as control (C) or  $10^{-7}$  M 4-OH tamoxifen (TAM) or  $10^{-7}$  M ICI (ICI) in SFPP medium as described in "Materials and Methods." Nuclear run-off assays for MDGI and  $\beta$ -actin were conducted as described in "Materials and Methods." The experiments were repeated three times with similar results.

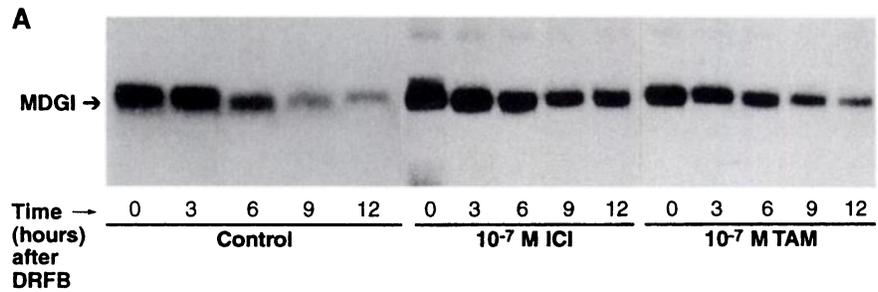
DRFB (Calbiochem, La Jolla, CA) as described (24). Total RNA was extracted from explants every 3 h after DRFB treatment and analyzed by Northern blotting using mouse MDGI cDNA (17). These blots were rehybridized with human  $\beta$ -actin cDNA probe as a control for inhibition of transcription as described (24).

**Immunoblot Analysis.** Explants were washed with PBS [140 mM NaCl, 2.5 mM KCl, and 15 mM  $\text{KH}_2\text{PO}_4$  (pH 7.2)] and homogenized in 2 $\times$  lysis buffer [1 $\times$  lysis buffer = 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 50 mM NaF, 2 mM phenylmethylsulfonyl fluoride, and 0.5% Nonidet P-40] for 30 s. The lysate was clarified by centrifugation at  $14,000 \times g$  for 15 min. Protein concentrations were determined as described (25), and 150  $\mu$ g of protein were electrophoresed on 16% SDS-polyacrylamide gels as described (20). Immunoblot analysis with a polyclonal rabbit antiserum against heart fatty acid-binding protein (26) at a 1:2000 dilution in Tris-buffered saline containing Tween 20 [20 mM Tris (pH 7.6), 137 mM NaCl, and 0.05% Tween 20] and horseradish peroxidase-conjugated antirabbit antiserum (Amersham, Oakville, Ont.) were performed using standard protocols. Western blots were visualized by a chemiluminescence-based photoblot system (ECL; Amersham).

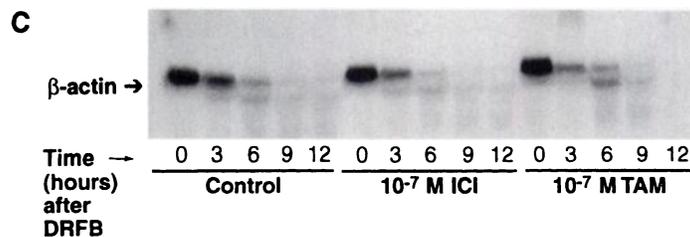
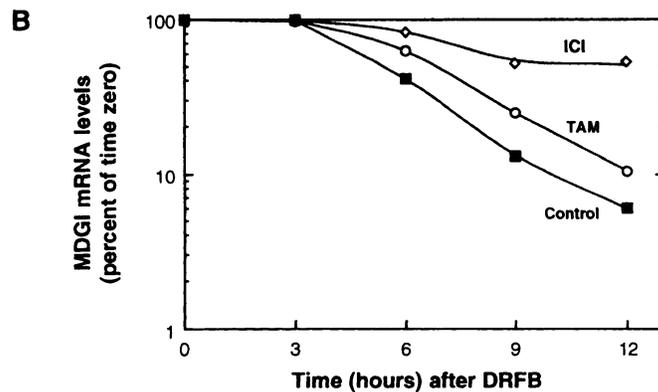
## RESULTS

**Effects of Antiestrogens on MDGI Gene Expression in Rat Mammary Explants.** Northern analysis revealed that tamoxifen and ICI had significant effects on MDGI mRNA abundance (Fig. 1). Densitometric scanning of Northern blots showed that MDGI mRNA abundance was more than 10- and 50-fold higher in mammary explants exposed to  $10^{-7}$  M 4-OH tamoxifen and  $10^{-7}$  M ICI, respectively, than it was in control explants (Fig. 1D). At equimolar concentrations, the potency of 4-OH tamoxifen in maintaining MDGI mRNA levels varied from 30 to 50% of that of ICI (Fig. 1D). There was no difference in MDGI mRNA levels when estradiol was added to mammary explant cultures (data not shown).

Extracts from mammary explants that were cultured in the presence of 4-OH tamoxifen and ICI were assayed for MDGI by



**Fig. 4** Effects of 4-OH tamoxifen and ICI on the half-life of MDGI mRNA. Fresh mammary explants were treated with either SFPF medium as control (C) or 10<sup>-7</sup> M 4-OH tamoxifen (TAM) or 10<sup>-7</sup> M ICI (ICI) in SFPF medium. mRNA stability assays were conducted as described in "Materials and Methods." The half-lives of MDGI mRNA (A) and  $\beta$ -actin mRNA (B) were determined as described in "Materials and Methods." The experiments were repeated three times with similar results. The half-life of MDGI mRNA (B) was estimated by densitometric scanning of Northern blots (A). The half-life of control  $\beta$ -actin mRNA was determined to be approximately 3 h, which is in agreement with a previous study (24).



Western blot analysis. Explants cultured in estrogen-free, SFPF medium showed low levels of MDGI protein, whereas tamoxifen and ICI treatments resulted in the accumulation of MDGI protein (Fig. 1C). Thus, the change in MDGI protein accumulation associated with 4-OH tamoxifen or ICI exposure was consistent with the change in MDGI mRNA levels. Thymidine incorporation was lower in ICI-treated than it was in 4-OH tamoxifen-treated explants (Fig. 1E), consistent with a negative correlation between MDGI accumulation, as estimated by Western blotting, and proliferation, as estimated by thymidine incorporation (data not shown).

Dose-response studies indicate that mammary explants completely lost the mRNA for MDGI in culture. ICI and, to a lesser extent, 4-OH tamoxifen were able to maintain MDGI mRNA levels (Fig. 2A). The MDGI levels observed in ICI-treated explants were about 60% of those seen in explants before they were cultured (Fig. 2C). These explants, obtained from pregnant rats (see "Materials and Methods"), expressed the high levels of MDGI previously associated with the

maximal differentiated function of the mammary gland during pregnancy (14).

A nuclear run-off assay demonstrated that neither ICI nor 4-OH tamoxifen affected the rate of *MDGI* gene transcription relative to that of controls (Fig. 3). Study of MDGI message stability using the mRNA specific transcription inhibitor DRFB demonstrated that the half-life for MDGI mRNA was approximately 6 h under control conditions. The half-lives were increased to about 7.5 h and more than 12 h following 4-OH tamoxifen and ICI treatments, respectively (Fig. 4). The half-life of control  $\beta$ -actin mRNA was determined to be approximately 3 h (Fig. 4C), which is in agreement with a previous study (24).

## DISCUSSION

Multiple effector pathways for antiestrogens action exist (3, 7, 22, 27-30). Data presented here provide the first evidence that, in addition to classic antiestrogen effects and more recently

described effects, such as up-regulation of TGF- $\beta$  (31) and modulation of IGF physiology at multiple levels (8, 9, 22, 27–29), antiestrogens up-regulate expression of *MDGI*, a tumor suppressor gene with relevance to the mammary gland (20, 21, 32). Furthermore, our studies show that antiestrogens produce a dose-dependent accumulation of MDGI in the mammary explant lysates by increasing the half-life of MDGI mRNA. Although we recognize that these are specific aspects of tamoxifen action, it is possible that the previously reported atrophic effects of tamoxifen on lobular structure (33) in humans may be due, in part, to up-regulation of MDGI.

Epidemiological data demonstrate that early first pregnancy is associated with a 2–5-fold life-long reduction in breast cancer risk (34, 35). The physiological basis for this protective early pregnancy effect remains unknown. One proposed mechanism is related to the effect of pregnancy on the differentiation of breast epithelial cells (36–38). Because *MDGI* is a tumor suppressor gene, the expression of which is related to breast epithelial differentiation (17, 20) and is up-regulated at the end of pregnancy (14), it is possible that protective effects of antiestrogen administration and of early pregnancy share a common mechanism that involves enhancement of MDGI expression.

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