

Androgens Down-Regulate *bcl-2* Protooncogene Expression in ZR-75-1 Human Breast Cancer Cells*

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ABSTRACT

Although a large proportion of primary human breast cancers express the androgen receptor, and treatment with androgens exerts beneficial effects in women with breast cancer, the role and especially the mechanism of action of androgens in breast cancer development and growth are not well understood. The potential effect of androgens on *bcl-2* protooncogene expression was investigated in a human breast cancer cell line whose proliferation is known to be inhibited by androgens. The estrogen-responsive ZR-75-1 cells were grown in the presence or absence of 5 α -dihydrotestosterone (DHT), alone or in

combination with 17 β -estradiol. DHT caused a marked down-regulation of Bcl-2 protein and messenger RNA levels in both the presence and absence of 17 β -estradiol. The inhibitory effect of DHT was completely prevented by coinubation with the pure antiandrogen hydroxyflutamide. The present data indicate that androgens can down-regulate *bcl-2* protooncogene levels via an androgen receptor-mediated mechanism, thus providing a novel mechanism for their known inhibitory effect on breast cancer cell growth. (*Endocrinology* 140: 416–421, 1999)

THE ANDROGEN receptor is expressed in 50–90% of breast tumors (1–3). Androgens or androgenic compounds, such as testosterone propionate (4–6), fluoxymesterone (7–9), and calusterone (10), have been shown to inhibit breast cancer growth in a proportion of women comparable to that achieved with other endocrine therapies. In fact, a direct growth inhibitory effect of androgens has been demonstrated in estrogen-responsive ZR-75-1 human breast cancer cells under both basal and estrogen-induced incubation conditions *in vitro* (11–19) as well as *in vivo* in nude mice (20). Other *in vitro* studies have shown that androgens can regulate the secretion of glycoproteins such as GCDFP-15 (12) and apolipoprotein D (18) as well as the expression of the estrogen receptor (13), but little is known regarding the mechanism of action of androgens on breast cancer cell growth.

The control of cell number is determined by a balance between cell proliferation and cell death. In multicellular organisms, apoptosis or programmed cell death represents a mechanism for the removal of unnecessary, aged, or damaged cells (for review, see Ref. 21). Cells from a variety of human cancers have a decreased ability to undergo apoptosis. Bcl-2 is an oncoprotein that acts by inhibiting programmed cell death and binds to several proteins that can participate in cell death regulation (for review, see Refs. 22 and 23).

To better understand the mechanism of androgen-induced growth inhibition in breast cancer, we examined the effect of 5 α -dihydrotestosterone (DHT) on Bcl-2 protein levels in ZR-75-1 human breast cancer cells. ZR-75-1 cells are growth inhibited by DHT, and they are also sensitive to the mitogenic effects of 17 β -estradiol (E₂), making them a unique model in which to study the antagonistic effects of androgens and estrogens in breast cancer (11, 12). The present data show that androgens down-regulate Bcl-2 expression in ZR-75-1 human breast cancer cells, thus offering a potential explanation for their inhibitory effect on cancer cell growth.

Materials and Methods

Chemicals

E₂ and DHT were purchased from Steraloids (Wilton, NH). The pure antiandrogen hydroxyflutamide was provided by Dr. Rudi Neri, Schering-Plough Corp. Research Institute (Kenilworth, NJ). The pure steroidal antiestrogen EM-139 was synthesized in the medicinal chemistry division of our laboratory (24, 25).

Cell culture

ZR-75-1 cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were plated in phenol red-free RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 IU penicillin/ml, 50 μ g streptomycin/ml, and (5% vol/vol) dextran-coated charcoal-treated FBS. After 72 h, the original medium was replaced with fresh medium of identical composition but containing, in addition, the indicated concentrations of steroids. Cells were then allowed to grow for the indicated time intervals. Culture medium was changed every 2–3 days. The cells were plated at densities such that confluence was not attained under any of the culture conditions.

Immunoblotting

Cultured cells were washed twice with cold PBS and then lysed on ice for 30 min in 50 mM Tris-HCl (pH 7.5), 0.25 M NaCl, 10% (vol/vol) Triton X-100, 0.1% (wt/vol) SDS, 0.5% (wt/vol) deoxycholate, 1 mM EDTA, the phosphatase inhibitors NaF (50 mM) and Na₃VO₄ (0.1 mM),

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and the following protease inhibitors: 0.1 mM phenylmethylsulfonylfluoride, leupeptin (1 $\mu\text{g/ml}$), soybean trypsin inhibitor (10 $\mu\text{g/ml}$), L-1-chloro-3-[4-tosylamido]-4-phenyl-2-butane (10 $\mu\text{g/ml}$), L-1-chloro-3-[4-tosylamido]-7-amino-2-heptasone-HCl (10 $\mu\text{g/ml}$), aprotinin (1 $\mu\text{g/ml}$), and 10 mM *N*-ethylmaleimide. Insoluble material was removed by centrifugation. Protein concentrations were measured using the Bio-Rad DC protein assay (Bio-Rad Laboratories, Inc., Hercules, CA). Proteins (15 $\mu\text{g/lane}$) were separated on 12% SDS-polyacrylamide gels and electroblotted to 0.2- μm nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH). The blots were probed separately with antibodies against Bcl-2 (MAb clone 124, Dako Corp., Carpinteria, CA), CPP32 (MAb C31720, Transduction Laboratories, Inc., Lexington, KY), Bcl-x (Pab B22630, Transduction Laboratories, Inc.), and pS2 (Pab NCL-pS2, NovoCastra Laboratories, Newcastle upon Tyne, UK), as indicated in the corresponding figures, followed by ECL-based detection (Amersham, Arlington Heights, IL). Each antibody recognized a single peptide of the expected size. Caspase-3 (CPP32), Bcl-2, and Bcl-x protein levels were quantified by scanning densitometry using the Chemilmager 400 (Alpha Innotech Corp., San Leandro, CA).

Immunohistochemistry

Cultured cells were harvested and washed twice with PBS. Cytospins were air-dried and fixed in 4% buffered formalin for 10 min. Bcl-2 was detected with the same antibody used for immunoblotting. Incubation with the primary antibody (90 min) was followed by incubation with biotinylated antimouse secondary antibody and peroxidase-conjugated streptavidin (Zymed Corp., South San Francisco, CA). The Bcl-2 antibody was visualized with 3,3'-diaminobenzidine tetrahydrochloride (5 mg/10 ml; Dako Corp.) to which 0.02% hydrogen peroxide was added just before use. Slides were counterstained with Gill hematoxylin and mounted with Permount (Fisher Scientific Corp., Fairlawn, NJ).

Ribonuclease protection assays

The Bcl-2 probe was constructed by cloning a *Pst*I-*Sph*I fragment (nucleotides 214–614 in the coding sequence) from pBS Bcl-2 (original Bcl-2 complementary DNA provided by Stanley J. Korsmeyer) into Bluescript SK vector. The plasmid was linearized with *Bst*X-1 for riboprobe synthesis.

The region corresponding to coding nucleotides 151–300 of the 15-kDa gross cystic disease fluid protein (GCDFFP-15) complementary DNA was obtained by PCR amplification using GCDFFP-15/pCMV-6 plasmid (provided by S. Gingras) as a template. The 150-bp fragment was subcloned in the Bluescript KS vector and sequenced. The recombinant plasmid was linearized with *Xba*I.

The 18S riboprobe was synthesized using the pT7 RNA 18S vector (Ambion, Inc., Austin, TX).

The complementary RNA probes were labeled with [α - ^{32}P]UTP (800 Ci/mmol) using T7 RNA polymerase and the Riboprobe *in vitro* Transcription System (Promega Corp., Madison, WI). The ribonuclease protection assay was carried out with 10 μg total RNA using the RPA-2 kit (Ambion, Inc., Austin, TX) according to the manufacturer's instructions. The products were analyzed by electrophoresis in 6% acrylamide-7 M urea gels. X-Ray films were quantitated by scanning densitometry using the Chemilmager 400 (Alpha Innotech Corp.) to measure Bcl-2 and GCDFFP-15 messenger RNA (mRNA) levels.

Results

Down-regulation of Bcl-2 protein levels by DHT

ZR-75-1 cells at passages 88–100 were cultured in stripped medium containing E_2 (10^{-10} M) alone or in combination with 1 nM DHT for 10 days. In eight separate experiments, including three that are presented in this report (Figs. 1, 2, and 5), we observed that Bcl-2 protein levels were, on the average, 57% lower (range, 47–68%) in cells that were incubated with DHT than in the corresponding control cells.

To determine the concentration of DHT that would produce a maximal decrease in Bcl-2 protein levels, ZR-75-1 cells were cultured in stripped medium containing 10^{-10} M E_2

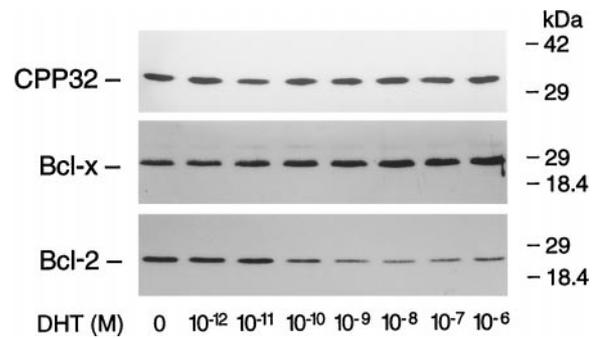


FIG. 1. Bcl-2 protein levels in ZR-75-1 cells incubated with increasing concentrations of DHT. ZR-75-1 cells were grown for 10 days in medium containing E_2 (10^{-10} M) alone or in combination with increasing concentrations of DHT from 10^{-12} – 10^{-6} M. Fifteen micrograms of total protein extract were separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-Bcl-2, anti-CPP32, and anti-Bcl-x antibodies.

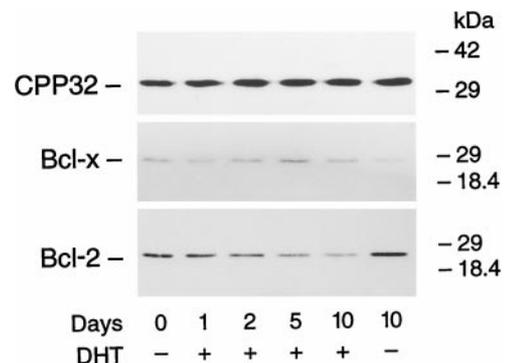


FIG. 2. Time course of DHT treatment on Bcl-2 expression in ZR-75-1 cells. ZR-75-1 cells were grown for 0, 1, 2, 5, and 10 days in medium containing E_2 (10^{-10} M) supplemented with DHT (10^{-9} M). The last lane represents cells grown for 10 days in the presence of E_2 alone. Fifteen micrograms of total protein extract was separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-Bcl-2 anti-Bcl-x and anti-CPP32 antibodies.

alone or in combination with increasing concentrations of DHT (10^{-12} – 10^{-6} M). The cells were harvested after 10 days of treatment, and Bcl-2 protein was detected by immunoblotting. As shown in Fig. 1, DHT caused a dose-related decrease in Bcl-2 protein levels. Based on densitometry data for Bcl-2 averaged from two separate dose-response experiments, Bcl-2 protein levels were 37%, 61%, and 64% lower in cells incubated with 10^{-10} , 10^{-9} , and 10^{-8} M DHT, respectively, than in control cells treated with E_2 alone. Higher concentrations of DHT did not cause a larger decrease in Bcl-2 protein levels. These results indicate that down-regulation of Bcl-2 by DHT occurs at a physiologically relevant concentration of DHT.

A survey of two other apoptosis-related genes whose products are detectable by immunoblotting in ZR-75-1 cells, namely CPP32 (caspase 3) and Bcl-x, did not reveal any modulation by DHT (Fig. 1). Similarly, protein levels of the cyclin-dependent kinase inhibitors p21 and p27 were unaffected by androgens (data not shown).

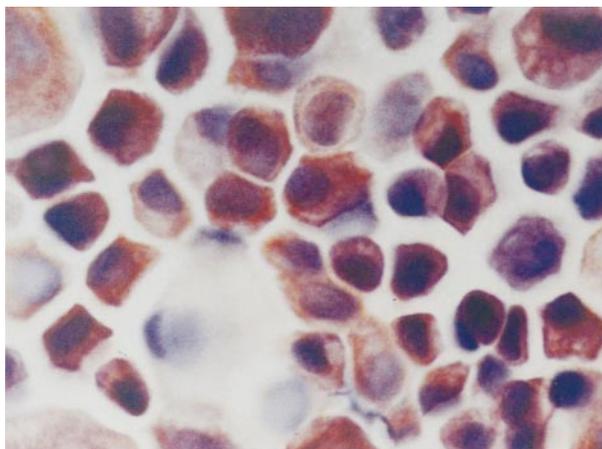
We then investigated the time course of the effect of a near-maximally effective concentration of DHT, namely 10^{-9} M, on Bcl-2 protein levels in ZR-75-1 cells. As illustrated in

Fig. 2, Bcl-2 protein decreased to its lowest levels at 5 days of exposure to DHT. Based on densitometry data averaged from two separate time-course experiments, Bcl-2 protein levels were 9%, 25%, 51%, and 53% lower in cells incubated with DHT for 1, 2, 5, and 10 days, respectively, than in control cells treated with E_2 alone. Bcl-x levels did not vary significantly over this time period.

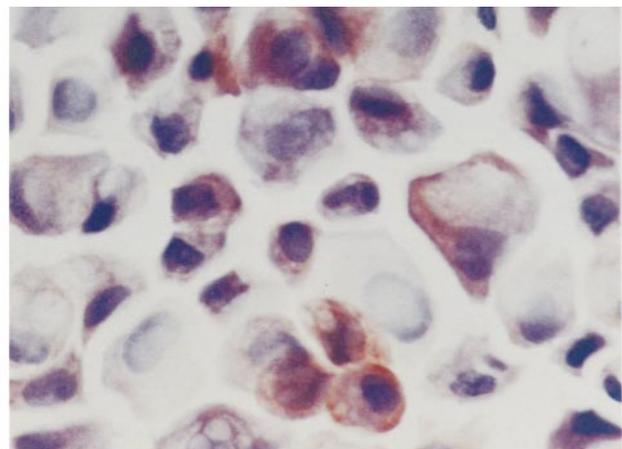
To confirm the results obtained by immunoblotting, we conducted an immunohistochemistry analysis of Bcl-2 in ZR-75-1 cells. As shown in Fig. 3, practically all of the ZR-75-1 cells cultured in stripped medium without E_2 showed strong cytoplasmic staining for Bcl-2. In contrast, a marked decrease in the intensity of Bcl-2 staining was observed when cells were treated for 10 days with DHT (10^{-9} M). In fact, only a few cells were positively stained for Bcl-2 after 10 days of exposure to DHT (Fig. 3).

Effect of DHT on Bcl-2 mRNA expression

To determine if the effect of DHT on Bcl-2 protein levels was attributable at least in part to an effect at the mRNA level, we examined the effect of DHT on Bcl-2 mRNA levels by ribonuclease protection assay. The riboprobe was designed to recognize both the Bcl-2 α and Bcl-2 β mRNA transcripts that result from an alternative splicing (25). RNA was isolated from ZR-75-1 cells cultured for 10 days in medium containing E_2 (10^{-10} M) alone or E_2 and DHT (10^{-9} M). As shown in Fig. 4 and confirmed by densitometric analysis, Bcl-2 α and Bcl-2 β mRNA levels were approximately 50% lower in DHT-treated cells than in cells treated with E_2 alone. 18S mRNA levels were similar in both RNA samples (data not shown). As a positive indicator of androgen action, we also examined the mRNA levels of the androgen-responsive GCDFP-15 gene in the same cells (12). As expected, DHT treatment caused an approximately 3-fold increase in GCDFP-15 mRNA levels.



CONTROL



DHT

FIG. 3. Immunohistochemical staining for Bcl-2 expression in ZR-75-1 cells treated with DHT. ZR-75-1 cells were cultured for 10 days in the absence (control) or presence of DHT (10^{-9} M). After treatment, cells were harvested for immunostaining with the anti-Bcl-2 antibody.

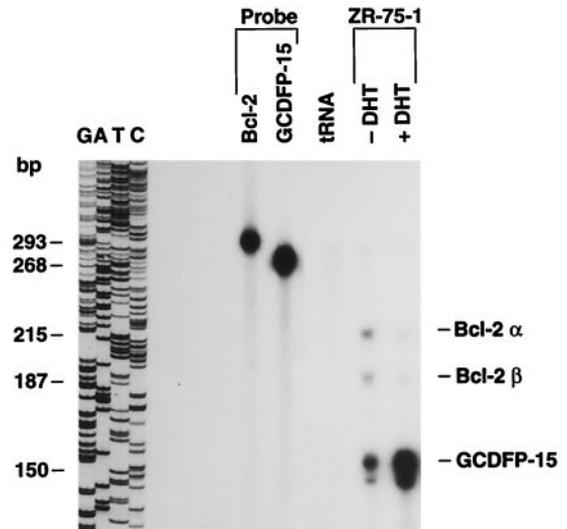


FIG. 4. Ribonuclease protection assay of Bcl-2 and GCDFP-15. *Upper panel*, Ten micrograms of total RNA from ZR-75-1 cells grown for 10 days in medium containing E_2 (10^{-10} M) alone or supplemented with 10^{-9} M DHT were hybridized with Bcl-2 and GCDFP-15 riboprobes. Yeast transfer RNA was used as a control for digestion. The length of the fragments was estimated using a DNA-sequencing reaction. *Lower panel*, The schematic portion of each probe corresponding to the coding sequence is shown as a dark rectangle.

Effects of estrogens and androgens on Bcl-2 protein levels in ZR-75-1 cells

As ZR-75-1 cells are sensitive to the effects of both estrogens and androgens, we next examined the effect of treatment with DHT and E₂ alone and in combination with the pure antiandrogen hydroxyflutamide or the pure antiestrogen EM-139, respectively, on Bcl-2 protein levels (Fig. 5). The levels of Bcl-2 protein in ZR-75-1 cells grown in stripped medium containing neither E₂ nor DHT served as the baseline.

In contrast to what has been observed in MCF-7 cells (Lapointe, J., and C. Labrie, personal data) (26), Bcl-2 protein levels were similar in control (Fig. 5, lane 1) and E₂-treated ZR-75-1 cells (lane 2). Moreover, Bcl-2 protein levels in E₂-treated cells were similar in the presence and absence of the antiestrogen EM-139 (compare lanes 2 and 7). The apparent lack of estrogenic regulation of Bcl-2 in ZR-75-1 cells was not attributable to a gross defect in estrogen-dependent transcription, because E₂ caused a marked increase in the protein levels of the estrogen-responsive pS2 gene that was completely blocked by EM-139 (compare lanes 1, 2, and 8). The slight decrease in Bcl-x levels observable in EM-139-treated cells (lane 7) was not reproducible.

DHT caused a decrease in ZR-75-1 Bcl-2 protein levels under both basal (in the absence of E₂) and E₂-stimulated conditions. Bcl-2 protein levels in cells treated with DHT were 61% lower than those in control cells (compare lanes 1 and 3). DHT also caused a 56% decrease in Bcl-2 levels in E₂-treated cells and completely antagonized the stimulatory effect of E₂ on pS2 expression (compare lanes 2 and 4). The pure antiandrogen hydroxyflutamide completely blocked down-regulation of Bcl-2 by DHT (lane 6). These results indicate that Bcl-2 is specifically regulated by an androgen receptor-mediated mechanism in ZR-75-1 cells.

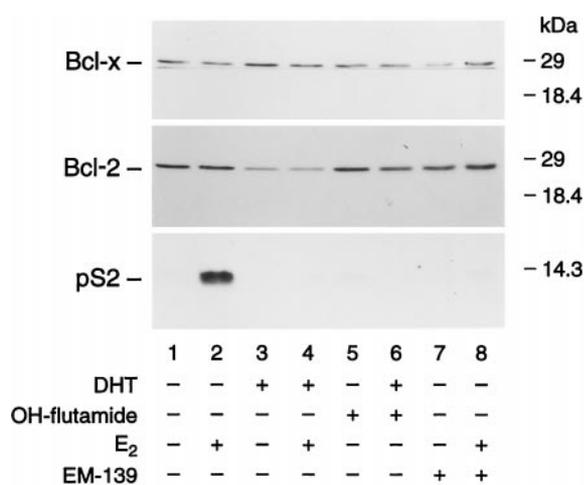


FIG. 5. Effects of androgen, estrogen, antiandrogen, and antiestrogen on Bcl-2 and pS2 protein levels in ZR-75-1 cells. ZR-75-1 cells were grown for 10 days with the following concentrations of hormones and antihormones: E₂, 10⁻¹⁰ M; DHT, 10⁻⁹ M; hydroxyflutamide, 10⁻⁶ M; and EM-139, 10⁻⁷ M, alone or in combination. Protein extracts were separated on SDS-PAGE and immunoblotted with anti-Bcl-2, anti-Bcl-x, and anti-pS2 antibodies.

Discussion

The *bcl-2* (B cell leukemia/lymphoma) gene was first discovered as the result of its location at the site of a 14;18 chromosomal translocation in follicular lymphomas (27). The t(14;18) locates the *bcl-2* gene from chromosome 18 next to the Ig heavy chain locus on chromosome 14, thus resulting in an increased production of the *bcl-2* gene product (28). *bcl-2* exerts its oncogenic function by blocking programmed cell death and extending B cell survival (29–32). Subsequent studies found that Bcl-2 expression is not restricted to lymphoproliferative malignancies, and the gene product could be identified in a number of normal nonhematolymphoid fetal and adult tissues. Bcl-2 is thus expressed in hormone-sensitive tissues, including the endometrium, prostate, and breast (33, 34). Immunohistochemically detectable Bcl-2 has been reported in breast carcinoma (35). In a series of 24 breast tumors, 96% of Bcl-2-positive tumors were estrogen receptor positive and 88% were positive for progesterone receptor (35).

The present data show for the first time that the androgen DHT down-regulates Bcl-2 protein and mRNA levels in ZR-75-1 breast cancer cells. Although DHT caused similar 50% decreases in both Bcl-2 protein and mRNA levels, the mechanism underlying DHT-dependent down-regulation of Bcl-2 is unknown at the present time. Additional experiments will be required to determine whether this is the result of a combination of effects at the mRNA and protein levels. It should be mentioned that there is no perfect consensus sequence for an androgen response element in the *bcl-2* gene sequence (28), thus suggesting that the mechanism(s) by which androgens regulate Bcl-2 expression may involve an indirect pathway(s). The relatively long period of exposure to DHT that is required for Bcl-2 down-regulation supports such a hypothesis.

The relative abundance of antiapoptotic proteins, such as Bcl-2, and proapoptotic proteins, such as Bak, is believed to play a critical role in maintaining the balance between cell life and death (22). It is therefore conceivable that the 50% decrease in Bcl-2 protein levels induced by DHT may be sufficient to disrupt the ratio between Bcl-2 and proapoptotic proteins, thereby rendering ZR-75-1 cells more susceptible to apoptosis. However, the control of apoptosis is far more complex, because other antiapoptotic proteins, such as Bcl-xL, and Mcl-1, may partially compensate for the decrease in Bcl-2 levels.

Nonetheless, it has already been demonstrated that variations in Bcl-2 levels can affect cell susceptibility to apoptosis. In fact, estrogen augments Bcl-2 levels in MCF-7 breast cancer cells, rendering them more resistant to the effects of adriamycin and taxol (26, 36). Overexpression of Bcl-2 alone is sufficient to protect breast and prostate cancer cells from apoptosis (26, 37), and conversely, down-regulation of Bcl-2 via antisense or ribozyme technology sensitizes MCF-7 and LNCaP cells, respectively, to apoptosis (26, 38). Thus, based on current knowledge, we would expect that a decrease in Bcl-2 levels would result in a greater susceptibility to apoptosis.

The mechanisms by which androgens and estrogens modulate Bcl-2 expression in hormone-sensitive tissues such as

the breast and prostate remain undetermined. Estrogens regulate Bcl-2 expression in breast cancer cells in a cell type-specific manner, as Bcl-2 is up-regulated in MCF-7 cells but not in ZR-75-1 cells (Ref. 26 and this report). The basis of this cell-specific regulation cannot be explained at the present time. Divergent results have also been obtained in the prostate. McDonnell *et al.* observed that castration augmented Bcl-2 mRNA levels in the basal cells of the rat prostate, suggesting that androgens down-regulate Bcl-2 expression in these cells (39). In human LNCaP prostate cancer cells, on the other hand, an approximately 50% increase in Bcl-2 mRNA levels was observed after 72 h of treatment with DHT (40).

DHT treatment markedly increased mRNA levels for the androgen-responsive GCDFP-15 gene in ZR-75-1 breast cancer cells, thus demonstrating the efficient activation of the androgen receptor by DHT in these cells. Moreover, Bcl-2 down-regulation was completely prevented by simultaneous treatment with hydroxyflutamide, a pure antiandrogen with an activity limited to blockade of the androgen receptor (41–45). These results indicate that DHT down-regulates Bcl-2 via an androgen receptor-mediated mechanism. It is of interest to note that DHT has been shown to down-regulate estrogen receptor mRNA expression in ZR-75-1 cells (13). In addition, up-regulation of Bcl-2 protein levels by estrogens has been observed in MCF-7 breast cancer cells (Lapointe, J., and C. Labrie, personal data) (26). However, this mechanism is unlikely to account for the effect of DHT on Bcl-2 expression in ZR-75-1 cells, as neither estradiol nor EM-139 modulated Bcl-2 protein levels, indicating that Bcl-2 expression is insensitive to estradiol in ZR-75-1 cells.

Previous data from our laboratory have shown that androgens and estrogens exert antagonistic effects on several parameters in breast cancer cells. For example, androgens block estrogen-induced cathepsin D mRNA expression (11, 12) and cell proliferation. The present data show that DHT also suppresses the estrogen-inducible pS2 gene in ZR-75-1 breast cancer cells. Moreover, the complete inhibition of pS2 expression by DHT was similar to the inhibition of pS2 achieved by the pure antiestrogen EM-139. The effect of DHT on Bcl-2 expression, however, does not seem to be related to the blockade of estrogenic action, because the effect of the androgen was observed in both the presence and absence of E_2 . Moreover, the antiestrogen had no effect on Bcl-2 expression despite its efficient blockade of estrogen action, as illustrated by the suppression of estrogen-stimulated pS2 expression and cell growth (data not shown).

In summary, the present study shows that androgens can down-regulate Bcl-2 gene expression. These findings provide new insights into the mechanisms of androgen action in breast cancer cells and help in understanding the beneficial effects of androgens observed on breast cancer in experimental models (7, 11, 12, 14–20) and in women treated with androgenic compounds (4–10).

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