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ANTAGONISM OF ESTROGEN- AND ANTIESTROGEN-INDUCED UTERINE COMPLEMENT COMPONENT C3 EXPRESSION BY ICI 164,384

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Summary—The uterus of the immature rat synthesizes and secretes complement component C3 in response to estradiol treatment. This response occurs in the uterine epithelial cells and is also stimulated by several antiestrogens including tamoxifen and LY117018. The administration of a new antiestrogen ICI 164,384 blocked the estradiol as well as the antiestrogen-stimulated increases in uterine weight, epithelial cell height, C3 synthesis and C3 mRNA. ICI 164,384 demonstrated no agonist properties in terms of epithelial cell response as determined by C3 expression.

INTRODUCTION

Estrogens have been implicated in the growth of some types of cancers including tumors of the breast and endometrium. Antiestrogens are an important group of drugs for the treatment of estrogen-dependent tumors [1]. Tamoxifen, the most commonly used antiestrogen, can behave as an estrogen agonist, a partial agonist, or as an antagonist [2]. Furthermore, since a significant number of estrogen receptor positive breast tumors do not respond to tamoxifen treatment [3] new antiestrogens have been developed.

One of the new antiestrogens, ICI 164,384 (ICI) appears to be an antagonist with no agonist properties in all systems investigated. Wakeling and Bowler, 1987 [4] reported that ICI is entirely devoid of estrogenic activity in the rat and mouse uterus and completely blocks the uterine stimulatory effects of estradiol and tamoxifen as measured by uterine weight. Wakeling and Bowler, 1988 [5] compared the effects of tamoxifen and ICI on the progesterone receptor concentration in the rat uterus and showed that, unlike tamoxifen, ICI does not induce the progesterone receptor and blocks induction of this receptor by estradiol. They further reported that ICI inhibits estradiol-induced growth of a breast cancer cell line (ZR-71-1) in a dose-dependent manner [4]. Studies by Weatherill *et al.*[6] demonstrated that ICI interacts directly with the estrogen receptor protein in a manner which prevents transformation of the complex to a form having increased affinity for DNA binding.

The purpose of the present study was to examine the antiestrogenic properties of ICI using a uterine epithelial cell specific response. Recent results from our laboratory and others have demonstrated that treatment of immature rats with estradiol results in the induction of C3 mRNA [7] and protein synthesis and secretion in the uterine epithelial cells [7, 8]. In this study we examine the effect of ICI on this cell-specific response.

EXPERIMENTAL

Animals

Immature female (21 days of age) Sprague–Dawley rats were obtained from Charles River (Farmington, Conn.). All animals were given free access to food and water and were maintained on a 14 h light and 10 h dark cycle. All injections were subcutaneous in saline:ethanol (50:50) and all controls were injected with vehicle alone. Estradiol was given at a dose of 1 μg, tamoxifen and LY117018 doses were 10 μg and the ICI doses was 100 μg unless otherwise noted.

In vitro incubations and protein analysis

Uteri were removed, split longitudinally and rinsed three times in a minimal essential medium (MEM) (Sigma, St Louis, Mo.) minus methionine and then incubated for 6 h at 37°C, in 5% CO_2 in MEM minus methionine containing penicillin (100 μg/ml), streptomycin (1 μg/ml) and 50 μCi/ml [^{35}S]methionine (1200 Ci/mmol, Amersham Inc., Arlington Heights Ill.). Incubation media were collected and cell and tissue debris removed by centrifugation. The

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[^{35}S]methionine incorporated into protein was determined by trichloroacetic acid precipitation and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) as previously described [9].

Complement component C3 synthesis was measured by immunoprecipitation. A volume of incubation medium containing 50,000 trichloroacetic acid precipitable CPM was incubated with 10 μg of goat anti-rat C3 antibody (Cappel, Organon Teknika Corp., West Chester, Penn). for 18 h at 4°C, following which 50 μl of washed Protein A (Pansorbin, Calbiochem Corp., LaJolla, Calif.) was added and incubated at room temperature for 1 h. The pellet

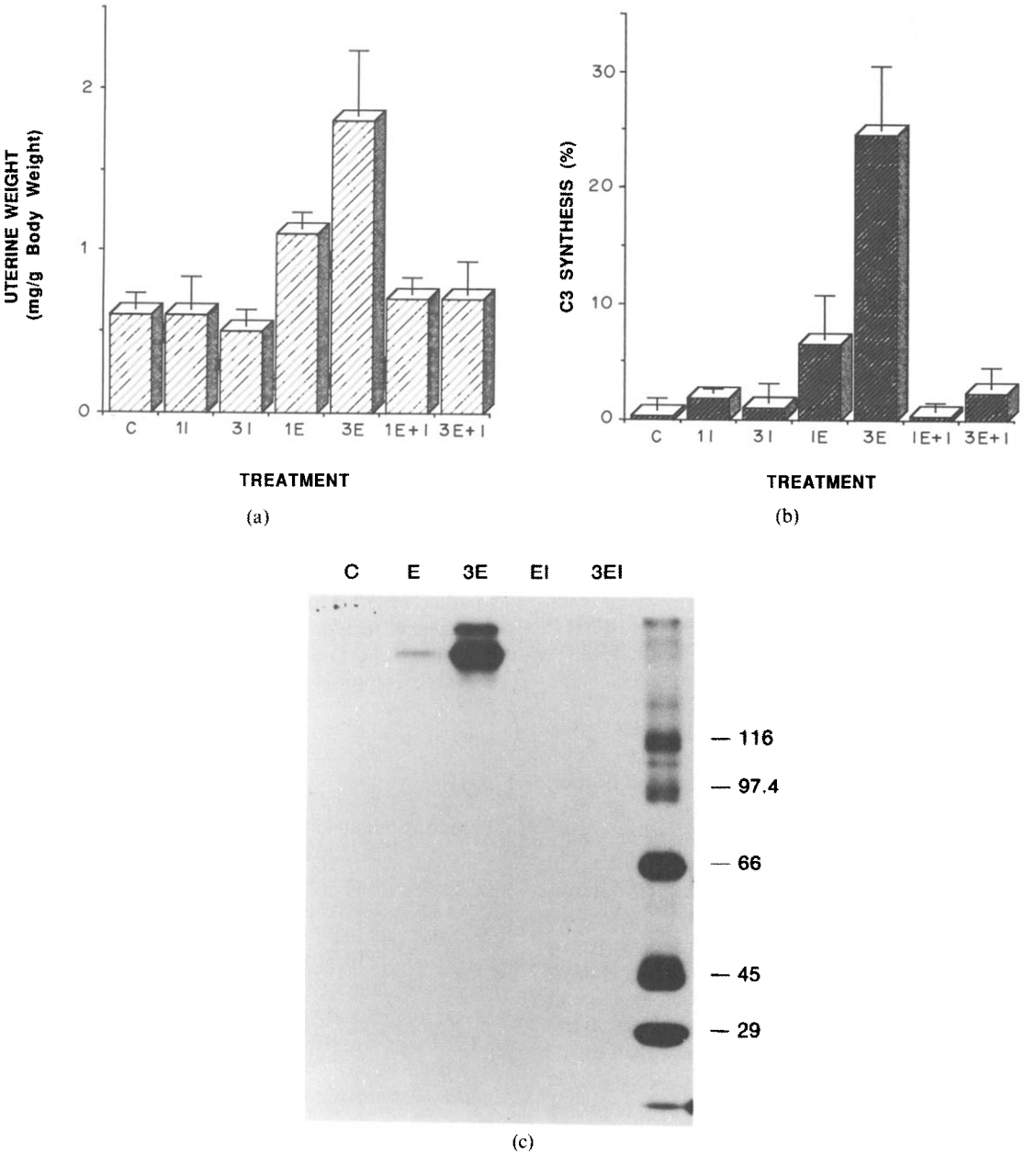


Fig. 1. Modulation of uterine weight and C3 synthesis by estradiol and ICI 164,384. Immature rats were injected with vehicle (C), 100 μg of ICI for either 1 day (1I) or 3 days (3I), estradiol 1 μg for 1(E) or 3 days (3E) or a combination of estradiol and ICI for 1 (1E + I) or 3 days (3E + I). Four animals were used per treatment. (a) Effect of E2 and ICI on uterine weight. (b) Effect of E2 and ICI on C3 synthesis. Data are mean ± SD and indicate C3 synthesis as a percentage of the total secretory protein synthesis. For both uterus/body weight and percent immunoprecipitation data only estradiol treatment resulted in a significant increase compared to controls ($P < 0.01$). Co-treatment with ICI and estradiol resulted in significant decreases compared to estradiol treatment alone ($P < 0.02$). (c) Fluorogram of the immunoprecipitated radiolabeled proteins.

obtained after a 5 min centrifugation at 10,000 *g* was washed by resuspension followed by centrifugation. This washing was repeated 2 times with Tris-buffered Saline (TBS) containing 0.5% Tween 20 and 0.05% SDS and once with TBS without these detergents. The washed pellet was resuspended in 100 μl of 2 × final sample buffer [10] without reducing agents, heated at 90°C for 10 min, centrifuged and a 10 μl aliquot of the supernatant counted in a scintillation counter. Results were expressed as percentages of total radiolabeled protein precipitated by anti-C3 antibody. Binding to goat anti-rat IgG antibody was subtracted from final percentages to correct for non-specific binding. The identity of the immunoprecipitated protein was confirmed by SDS–PAGE by running 50 μl of each immunoprecipitated sample.

Histology

Uterine sections were fixed in neutral buffered formalin for 24 h then rinsed and stored in 95% ETOH until further processed as previously described [11].

RNA isolation and analysis

RNA was extracted from uterine tissue and messenger RNA isolated using methods previously described [12]. Equal amounts of mRNA (2 μg) were separated in 1% agarose gels containing 2.2 M formaldehyde, transferred electrophoretically to Nytran (Schleicher & Schuell, Keene, N.H.) [13] and hybridized to nick translated cDNA probes [14]. The cDNA probes used were LE-1, a C3 cDNA [7] and 1A, a non-regulated cDNA [15].

Statistics

The Student's *t*-test with a significance level of $P < 0.05$ (Statworks, Cricket Software, Malvern, Penn.) was used for statistical analysis of data.

RESULTS

The co-administration of ICI and estradiol to immature rats completely prevented the estradiol stimulated increase in uterine wet weight (Fig. 1a). In agreement with the results of Wakeling and Bowler [4], ICI alone, for either one or three days, did not result in any significant increase in uterine wet weight.

The administration of estradiol to immature rats stimulates the secretion of radiolabeled C3 [7, 8]. The estradiol stimulated synthesis and secretion of C3 was blocked by co-treatment with ICI (Fig. 1b). Figure 1c confirms that the immunoprecipitated radiolabeled protein migrated as a single 180 kDa band, characteristic of non-reduced C3 [7, 16]. Furthermore, no radiolabeled proteins were immunoprecipitated following co-treatment with estrogen and ICI. ICI alone did not induce any secretion of radiolabeled C3. This pattern of C3 regulation very closely matched the pattern of uterine weight gain as seen in Fig. 1a.

The estradiol stimulated synthesis of C3 has been shown to occur exclusively in the luminal epithelial (LE) cells of the rat uterus [7, 17]; therefore, we examined the effects of the co-administration of ICI and estradiol on the height of the LE cells. The hematoxylin and eosin stained sections shown in Fig. 2a and b demonstrate the dramatic antagonist effect of ICI on the estradiol-stimulated increase in the height of the LE cells. Furthermore, several studies have reported that several antiestrogens, functioning as agonist or partial agonists, increase epithelial cell height [18, 19]. Similarly, ICI antagonized the tamoxifen-stimulated increase in epithelial cell height (Fig. 3a and b). These differences were quantitated by measuring uterine luminal epithelial cell heights in animals treated with estradiol, tamoxifen, or LY117018 alone or in combination with ICI (Table 1).

Coincident with their ability to increase uterine epithelial cell height several antiestrogens including tamoxifen, LY117018, and LY156758 have been shown to increase C3 synthesis by the epithelial cells [17, 20]. The increased synthesis of C3 stimulated by 10 μg of either tamoxifen or LY117018 was prevented by the administration of ICI as measured by immunoprecipitation with anti-C3 antibodies (Fig. 4a). Additionally, for the tamoxifen response, ICI also blocked at doses of 50 or 100 μg. The immunoprecipitated radiolabeled proteins were

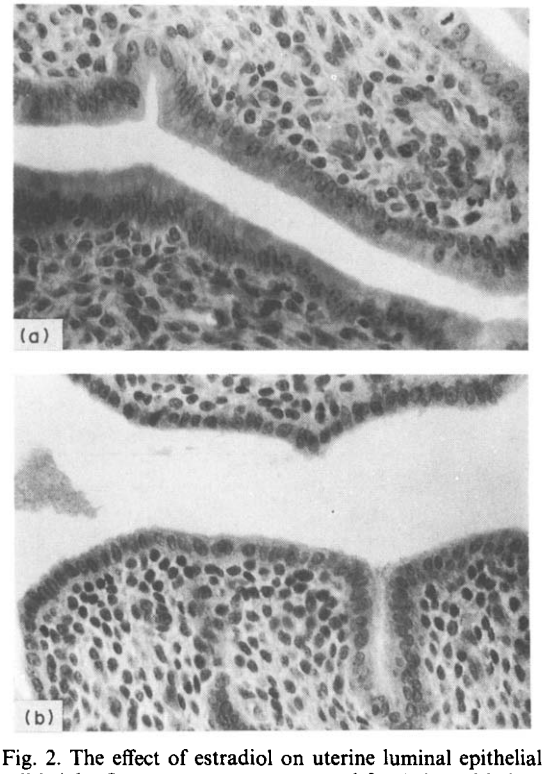


Fig. 2. The effect of estradiol on uterine luminal epithelial cell height. Immature rats were treated for 1 day with 1 μg of estradiol (a) or with estradiol and 100 μg of ICI 164,384 (b). Sections were stained with hematoxylin and eosin.

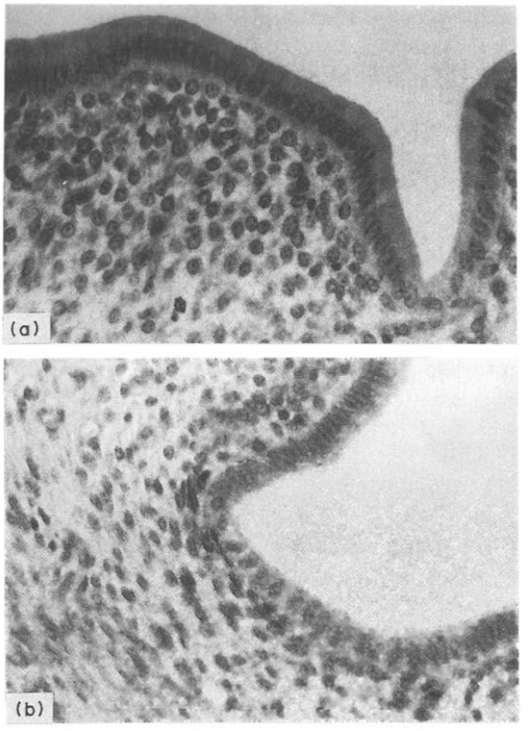


Fig. 3. The effect of tamoxifen on uterine luminal epithelial cell height. Immature rats were treated for 1 day with 10 μg of tamoxifen (a) or with tamoxifen and 100 μg of ICI 164,384 (b).

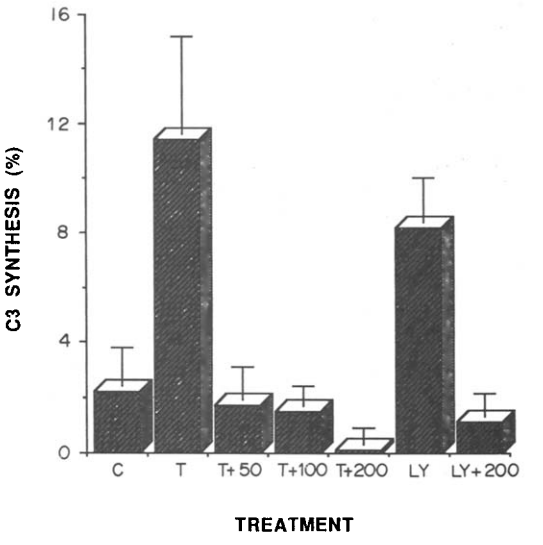
examined by SDS–PAGE and demonstrate that a single protein was present after treatment with tamoxifen or LY117018 alone and was not detected when ICI was coadministered.

The estradiol-induced increase in C3 synthesis and secretion has been shown to result from an increase in C3 mRNA concentration [7]. The effects of ICI on estradiol-induced C3 mRNA were examined in order to determine the site of regulation. ICI was able to antagonize the estradiol-induced increase in the concentration of C3 mRNA (Fig. 5A). Treatment with ICI alone resulted in no increase in the concentration of C3 mRNA compared to vehicle treated control rats (data not shown).

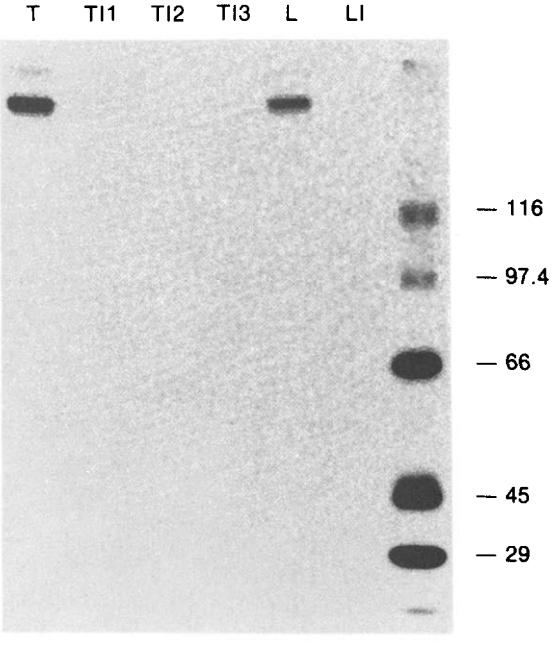
Table 1. Rat uterine luminal epithelial cell height

Treatment	Cell height (μm)
Con	13.2 ± 1.5
ICI	13.7 ± 1.4
E2	20.3 ± 0.9
E2 + ICI	14.0 ± 0.3
Tam	23.3 ± 0.9
Tam + ICI	15.4 ± 0.4
LY	19.7 ± 0.7
LY + ICI	16.0 ± 0.4

Treatments with estradiol, tamoxifen, or LY117018 significantly increased cell height compared to control ($P < 0.002$). Co-administration of ICI with estradiol, tamoxifen, or LY117018 significantly decreased height compared to the agonist alone ($P < 0.001$). Statistics are based on 3 uteri per treatment group.



(a)



(b)

Fig. 4. Modulation of uterine C3 synthesis by tamoxifen, LY117018 and ICI 164,384. (a) Immature rats were treated for one day with vehicle (C), 10 μg tamoxifen alone (T) or tamoxifen co-administered with either 50 (T + 50), 100 (T + 100) or 200 (T + 200) μg of ICI 164,384. Four rats were used per treatment group. Animals were also treated with 10 μg of LY117018 alone (LY) or LY117018 co-administered with 200 μg of ICI (LY + 200). The data are expressed as a percentage of the total secretory protein synthesis (mean ± SD). Only tamoxifen (T) and LY117018 (LY) alone were significantly greater than control ($P < 0.002$). Co-treatment of tamoxifen or LY117018 and ICI resulted in significant decreases compared to tamoxifen or LY117018 alone ($P < 0.02$). (b) Fluorogram of the immunoprecipitated proteins from (a) analyzed on a 7.5% acrylamide SDS–PAGE.

DISCUSSION

The data in this paper clearly demonstrate that the estradiol stimulated increases in uterine wet weight, epithelial cell height, C3 synthesis and secretion as well as C3 mRNA concentration were completely prevented by the co-administration of ICI. This effect appears to occur using a 100:1 ratio of antihormone to hormone. Furthermore, this compound blocked both C3 expression and epithelial cell height stimulated by the antiestrogens tamoxifen and LY117018. Thus ICI was able to prevent hormone as well as antihormone induction of gene expression. Additionally, in contrast to tamoxifen, the administration of ICI alone was completely devoid of estrogenic activity. These results agree and extend those of Wakeling and Bowler [4, 5] by demonstrating the ability of ICI to block an estrogen-induced uterine epithelial cell specific response.

Tamoxifen and LY117018 as well as other antiestrogens have been shown to prevent the estradiol stimulated increase in uterine peroxidase activity [21, 22]. This increased peroxidase activity is predominantly due to eosinophil infiltration [23, 24] caused by estrogen stimulation of an Eosinophil Chemotactic Factor in the Uterus (ECF-U) [25]. The estradiol stimulation of ECF-U is prevented by the co-administration of tamoxifen and is not modulated by tamoxifen alone. In contrast, tamoxifen induces C3 expression in the LE cells and is unable to antagonize estradiol induction [20]. Thus tamoxifen and LY117018 and several other antiestrogens function as antagonists on one uterine response and function as agonists on another. The induction of C3 synthesis by antiestrogens is delayed approximately 12 h when compared with the estradiol induction [20]. Similar delays have been reported in the stimulation of ornithine decarboxylase activity and are related to dose and route of administration [26]. This however does not appear to be the case for the antiestrogen stimulation of C3 induction [20]. The lack of induction of C3 expression by ICI is not likely due to a prolonged delay in response since even following three days of treatment ICI was a true antagonist.

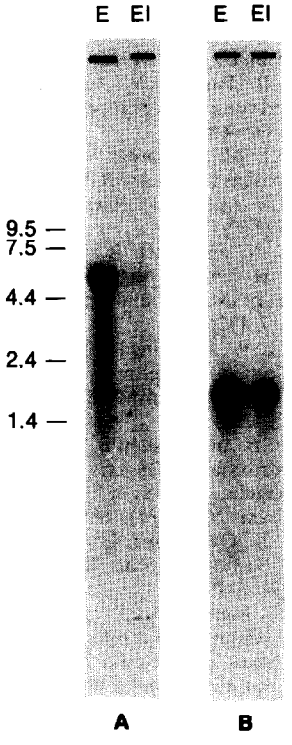


Fig. 5. Effect of Estradiol and ICI 164,384 on uterine C3 mRNA. Equal concentrations of poly A^+RNA isolated from rats treated for one day with 1 μg of estradiol (E) or estradiol co-administered with 100 μg of ICI 164,384 (EI) were hybridized with nick-translated LE-1 cDNA (A). The blot was stripped and re-hybridized with 1A cDNA in order to demonstrate that equal amounts of mRNA were applied to each well (B).

The underlying assumption of antiestrogen action is that they function through interaction with the estrogen receptor. Although data have been presented for the existence of antiestrogen binding sites further evidence demonstrates that these compounds appear to function through the estrogen receptor. The possible role of antiestrogen binding sites, if any, in antihormone action remains to be clearly established [27].

The differences in biological activity of various antiestrogens may be related to their binding affinity to the estrogen receptor; for example, the affinities for the rat estrogen receptor are as follows; ICI > OH-Tam > Tam [28]. While the binding affinity may be important, the conformational structure of the antiestrogen as described and modeled by Jordan and Koch must be considered [29]. The different responses to antiestrogens in the same tissue (ECF-U and C3 synthesis) or in the same cell [30, 31] may be explained by the presence of cell specific factors which could interact with the ligand-receptor complex. Preliminary data suggest that the ECF-U is localized in the stromal cells (unpublished observations) whereas C3 expression occurs in the epithelial cells. However, recent reports indicate that the accumulation of calbindin-D_{9k} stimulated by tamoxifen occurs only in the myometrial and stromal cells and not in the epithelial cells [32]. Thus tamoxifen does not appear to demonstrate a consistent cell-specific response in the rat uterus. Another possibility is that antiestrogens may act indirectly through the expression of growth factors which may control specific gene expression in either a positive or negative fashion. Such modulation of growth by autocrine growth factors has been shown to occur in MCF-7 cells [32–34].

The availability of an antiestrogen with no agonist properties may be of value in the treatment of hormone responsive tumors as well as providing a useful tool to elucidate the mechanisms of hormone and antihormone regulation of gene expression.

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