Comparative Studies of Aromatase Inhibitors in Cultured Human Breast Cancer Cells

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Abstract

The presence of aromatase activity, estrogen receptors, and estrogenic responsiveness in MCF-7 human breast cancer cells has allowed this cell line to be used as a unique in vitro system for investigating the biological activities of potentially therapeutic aromatase inhibitors. We now report the results of studies which have examined the cytotoxicity, antiaromatase, and intrinsic estrogenic activities of aminogluthethimide, 1,2-dehydrotestolactone (testolactone), dihydrotestosterone, 4-hydroxy-4-androstene-3,17-dione, and 10-propargylestr-4-ene-3,17-dione within MCF-7 monolayer cultures. Cell viability was determined by trypan blue exclusion, and aromatase activity was assessed by quantifying the amounts of [3H]estradiol formed from [3H]testosterone. Estrogenic activity was assessed by examining the ability of each inhibitor to increase cytoplasmic progesterone receptor and deplete cytoplasmic estrogen receptor concentrations in these cells during a 5-day incubation period. Cytoplasmic progesterone and estrogen receptors were measured by the single-saturating-dose technique using [17α-methyl-3H]17α,21-dimethyl-19-norpregna-4,9-diene-3,20-dione and [3H]estradiol as the labeled ligands for each assay, respectively. The results showed that all of these compounds were noncytotoxic aromatase inhibitors in MCF-7 cells but that these agents demonstrated marked differences in inhibitory potency (10-propargylestr-4-ene-3,17-dione > 4-hydroxy-4-androstene-3,17-dione > dihydrotestosterone > testosterone = aminogluthethimide). The incubation of cells with 4-hydroxy-4-androstene-3,17-dione resulted in cytoplasmic progesterone and estrogen receptor responses that were similar in magnitude to those observed in other cultures incubated with equimolar concentrations of estradiol. None of the other four agents demonstrated estrogenic activity in this system. However, we have previously observed that dihydrotestosterone has substantial antiestrogenic action in this system. Taken together, these results indicate that some aromatase inhibitors may influence the hormonal regulation of human breast cancer cells by more than one mechanism.

Introduction

The identification of estrogen receptors (5), estrogenic responsivenes (6, 7), and aromatase activity (8) within MCF-7 human breast cancer cells has suggested that these cells may provide a useful model for investigating both the regulation and the metabolic impact of intracellular estrogen biosynthesis within hormone-dependent human breast cancers. In pursuing this line of investigation, our laboratory has taken the opportunity to examine the biological activities of several putative aromatase inhibitors within this system. In this report, we present the results of studies which have examined the cytotoxic, antiaromatase, and estrogenic activities of AG, TES, DHT, 4OHA, and PED.

Materials and Methods

Silica gel thin-layer chromatography sheets (Sil G-H) were purchased from Brinkmann Instruments, Inc. (Des Plaines, Ill.), and Celite Analytical Filter-aid was obtained from Johns-Manville Products Corp. (Lompoc, Calif.). Anhydrous ethanol, isooctane, ethylene glycol, and ethyl acetate were purchased from Fisher Scientific Co. (Fair Lawn, N.J.); methanol, ethanol, and acetone were from J. T. Baker Chemicals Co. (Phillipsburg, N.J.), and TES was supplied by E. R. Squibb and Sons, Inc. (Princeton, N.J.). PED was prepared as described previously (4), and 4OHA was synthesized by the methods described by Brodie et al. (3). DHT and other unlabeled steroids were obtained from Steraloids, Inc. (Wilton, N.J.), and recrystallized 3 times in acetone:water before use.

Cell and Culture Techniques. Seed cultures of MCF-7 cells were originally obtained from the Michigan Cancer Foundation (Detroit, Mich.) and have been maintained as described previously (8). Experimental cultures were grown in 150-sq cm plastic flasks in a 20-ml volume of growth medium consisting of Swim’s S-78 medium with l-glutamine and NaHCO3 (Grand Island Biological Co., Grand Island, N.Y.) supplemented with CaCl2 (2.5 mM), l-cystine (50 µM), N-tris(hydroxymethyl)methylyglycine buffer (0.1 M), bovine insulin (0.6 ng/ml), and both 5% calf and 5% fetal calf sera. Experimental agents were added to each flask in 20 µl ethanol. Cultures received fresh medium every 24 hr. Cells were harvested with 0.1 M phosphate-buffered saline (0.1 M NaCl, 1.5 mM KH2PO4, 7.7 mM Na2HPO4, 2.7 M KCl, pH 7.6), washed twice, and counted with a hemocytometer. Cell viability, as assessed by the trypan blue exclusion technique, was observed to be at least 85% for all experiments.

Aromatase Assay. MCF-7 aromatase activity was measured by quantifying the amounts of [3H]estradiol formed from [3H]testosterone within individual subconfluent monolayer cultures. Although the details of our original assay are described elsewhere (8), the current experi


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ments were performed using several modifications of those techniques. Each culture was incubated with 30 nm [3H]testosterone and 10 nm estradiol (estrogen trap), in the presence or absence of various concentrations of test agents for 6 hr at 37°. Medium control flasks containing medium, [3H]testosterone, and estradiol, but no cells, were included in each study. Following incubation, the cells from each flask were harvested and counted, and the medium from each culture was assayed for [3H]estradiol. Medium was extracted twice with ether following the addition of approximately 10° cpm [3H]estradiol to each sample. These extracts were dried, extracted with dichloromethane to separate the steroids of interest from more polar lipids, dried again, and cochromatographed with unlabeled estradiol on plastic-backed silica gel thin-layer chromatography sheets using 2 ascents of a dichloromethane:ethyl acetate (90:10, v/v) solvent system. The estradiol-containing spots in each lane were then identified with UV, cut out, and recrystallized 6 times with acetone:water solvent pair. Constant [3H]:[14C] ratios were achieved in each sample by the third recrystallization. The measurement of radioactivity in each sample and the determinations of [3H]estradiol content within the original medium samples were carried out by methods described elsewhere (8, 11).

PR Concentrations. PR was assayed in cytosol preparations using the synthetic progestin, R5020, as ligand. The abilities of unlabeled test agents to interfere with the receptor assay by examining the abilities of each agent to compete with [3H]-R5020 for PR binding. Sets of triplicate cytosol samples from estrogen-stimulated cells were incubated with 5 nm [3H]-R5020 and 0.01 to 1 μM concentrations of each aromatase inhibitor or unlabeled R5020 for 19 hr at 4°. The amounts of specific [3H] binding activity in each tube were then compared to those values observed in the absence of competing ligand. The results (Chart 2) revealed that none of the 5 aromatase inhibitors were effective binding competitors under these conditions.

Sets of triplicate subconfluent cultures were then incubated with growth medium alone (basal flasks) or 0.01 nm to 1 μM AG, TES, DHT, 4OHA, PED, or estradiol for 5 days. PR concentration, cultures were grown for 5 days in the presence or absence of each test agent. Cytosol was then prepared from the washed cell pellets from each control and experimental flask and assayed for PR by the single-saturating-dose technique using 20 nm [3H]R5020 ± 1 μM unlabeled progesterone. The technical details, comparative reliability, and both the intra- and interexperimental variability of this technique have been reported elsewhere (10).

ER Determinations. ER determinations were carried out in MCF-7 cytosol preparations using labeled and unlabeled estradiol as ligands. ER binding competition studies were carried out as described elsewhere (12). The effects of various test agents upon basal MCF-7 ER concentration were examined using cells grown in the presence of each agent for 5 days. Cytosol samples were assayed for ER content by the single-saturating-dose technique using 15 nm [3H]estradiol ± 1 μM unlabeled estradiol. Incubations were carried out at 4° for 19 hr, and dextran-coated charcoal was used to separate free and bound steroids.

Protein Assay. Aliquots of each cytosol preparation were assayed for protein content by the method of Bradford (2).

Results

Effects of AG, TES, DHT, 4OHA, and PED on MCF-7 Cell Viability and Aromatase Activity. The cytotoxicity of each putative aromatase inhibitor was examined by incubating sets of triplicate confluent MCF-7 cultures with 0 to 1 μM concentrations of each agent for 5 days. The numbers of total and viable cells observed in each experimental flask were within 90% of the control flask values, indicating the absence of detectable cytotoxicity for each agent under these conditions (data not shown).

The aromatase-inhibitory activities of these compounds were assessed by comparing the amounts of [3H]estradiol formed from [3H]testosterone by subconfluent cultures during a 6-hr incubation at 37° in the absence and presence of each agent. The results (Chart 1) indicated that all 5 compounds were capable of suppressing estradiol biosynthesis but also showed a substantial variation between the apparent biological potencies of these agents. AG and TES were completely ineffective at concentrations up to 1 nm, and the concentrations of each agent required to inhibit aromatase activity by 50% were calculated to be 400 and 300 nm, respectively. A comparison of the 50% aromatase activity-inhibitory concentration values for these agents revealed TES, DHT, 4OHA, and PED to be approximately 1.3, 57, 4000, and 8000 times more potent inhibitors than was AG.

Effects of Aromatase Inhibitors upon MCF-7 PR. One specific effect of estrogens in MCF-7 cells is the stimulation of PR synthesis (6). We have examined the intrinsic estrogenic activity of AG, TES, DHT, 4OHA, and PED by investigating the effect of each agent upon basal PR concentration. Before undertaking these experiments, however, we first tested the ability of these compounds to interfere with the receptor assay by examining the abilities of each agent to compete with [3H]-

[3H]estradiol for PRC binding. Sets of triplicate cytosol samples from estrogen-stimulated cells were incubated with 5 nm [3H]-R5020 and 0 to 1 μM concentrations of each aromatase inhibitor or unlabeled R5020 for 19 hr at 4°. The amounts of specific [3H] binding activity in each tube were then compared to those values observed in the absence of competing ligand. The results (Chart 2) revealed that none of the 5 aromatase inhibitors were effective binding competitors under these conditions.

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Protein Assay. Aliquots of each cytosol preparation were assayed for protein content by the method of Bradford (2).

Chart 1. Effects of AG (●), TES (●), DHT (●), 4OHA (●), and PED (●) on aromatase activity in MCF-7 cells. Cultures were incubated with 30 nm [3H]-testosterone, 10 nm unlabeled estradiol, and either increasing concentrations of each inhibitor or no added drugs (control flasks) for 6 hr at 37°. The amounts of [3H]estradiol formed during each incubation were measured by the techniques described under "Materials and Methods" and expressed as a percentage of the control flask values. The amounts of [3H]estradiol observed in cell-free growth medium-incubated [3H]testosterone for the same period have been subtracted from each culture value. Each value represents the mean of triplicate culture determinations.
dose-dependent increases in PRc that were similar in magnitude to the receptor responses observed following treatment with equimolar concentrations of estradiol. Of further interest was the observation that higher concentrations of either 4OHA or estradiol were associated with progressive decreases in PRc toward basal culture values.

**Interaction of 4OHA with MCF-7 ERc.** Since the expression of estrogenic activity in responsive target organs involves the translocation of ERc to the nuclear compartment, we examined the ability of 4OHA and estradiol treatments to deplete cytoplasmic receptor concentrations in MCF-7 cells. Sets of triplicate subconfluent cultures were incubated for 5 days with growth medium alone (basal flasks) and 0 to 10 nm 4OHA or estradiol. The results of these experiments revealed that treatment with either steroid was associated with dose-dependent decreases in cytosol receptor concentration (Chart 4). To further explore the interaction of 4OHA with MCF-7 ERc, we compared the abilities of increasing concentrations of 4OHA and unlabeled estradiol to compete with [3H]estradiol for estrogen-binding sites in MCF-7 cytosol. Although 1 μM estradiol completely inhibited the specific binding of labeled ligand, a similar concentration of 4OHA reduced [3H]estradiol binding activity by less than 35% under these conditions.

**Discussion**

The data presented in this report suggest that neither AG, TES, AG, 4OHA, nor PED exhibit significant cytotoxicity in MCF-7 cells but that each of these agents can inhibit the intracellular biosynthesis of estrogens within these hormone-responsive human breast cancer cells. Although AG and TES represent the least potent of the aromatase inhibitors tested, data supporting the abilities of both agents to induce objective tumor regression in some patients with hormone-responsive breast cancers are reported elsewhere in this supplement. Since one of the effects of AG and the only currently known action of TES is to inhibit aromatase activity, it is interesting to speculate that the therapeutic efficacy of both these agents is a direct result of their abilities to decrease circulating and/or intracellular concentrations of estrogens. The results of our studies also indicate that the aromatase-inhibitory activity of DHT is nearly 60-fold greater than that observed for AG in this system. Furthermore, DHT has been shown to exert substantial antiestrogenic activity in MCF-7 by mechanisms that appear to be unrelated to its antiaromatase effect (9, 10). Although the potent virilizing effects of this androgen detract from its clinical usefulness in female patients, the combined actions of DHT suggest that this androgen may provide a highly effective treatment for hormone-responsive breast cancers in men.

To our knowledge, this report provides the first available information concerning the biological activities of either 4OHA or PED in a hormone-responsive human breast cancer cell system. PED, a “suicide” aromatase substrate in other systems (4), represented the most potent inhibitor of estradiol synthesis.
in MCF-7 cells of the 5 agents tested. This feature, combined with the undetectable cytotoxic and estrogenic activities of PED, suggests that this agent may prove to be of clinical value in treating hormone-responsive tumors in both women and men.

Perhaps the most interesting aspect of these studies is the finding that 4OHA is capable of exerting both potent antiaromatase action and substantial estrogenic activity in MCF-7 cells. This fascinating dichotomy underscores the usefulness of systems such as MCF-7 in examining potential human breast cancer therapies for unanticipated biological actions. The results of the ERc competition study (Chart 5) essentially exclude the possibility that the induction of PRc observed following 4OHA treatment was due to the presence of contaminating estradiol in our 4OHA preparation. However, the limited ability of 4OHA to compete with estradiol also suggests that this aromatase inhibitor binds rather poorly to MCF-7 ERc under these conditions. However, the exposure of intact cells to this agent resulted in both dose-dependent increases in PRc and decreases in ERc that were similar to the receptor responses observed in cells exposed to equimolar concentrations of estradiol. This finding provides strong support for the concept that the stimulation of PRc observed with 4OHA treatment is mediated by an estrogen receptor mechanism. The apparent discrepancy between the results of the cytosol (Chart 5) and intact cell (Chart 4) studies suggests either that the interaction between 4OHA and MCF-7 ERc varies substantially between the 2 test systems or that the estrogenic activity of 4OHA within intact cells is mediated by the intracellular conversion of this steroid to a more potent estrogenic metabolite. We currently favor the latter hypothesis since major differences in hormone-binding affinity between these systems have not been reported previously and since 4OHA does not appear to have estrogenic activity within rat uterus or vagina (3).

Although 4OHA can stimulate the induction of MCF-7 PRc, the effects of this agent upon estrogen-dependent cell growth remain to be clarified. It is interesting to note, however, that the biphasic effect of increasing 4OHA or estradiol concentrations upon MCF-7 PRc bears a striking similarity to the paradoxical effects of physiological and pharmacological estrogen doses upon the growth of hormone-dependent human breast tumors. If both phenomena were to be mediated by similar mechanisms, one might predict that low concentrations of 4OHA, like estradiol, might augment the growth of such tumors, whereas high doses might result in tumor regression. Brodie et al. (3) have clearly demonstrated that relatively large doses of 4OHA reduce circulating estrogen levels and result in the regression of hormone-dependent experimental breast tumors in female rats. These effects may have resulted from the antiaromatase action of 4OHA alone. However, our data suggest the alternative possibility that the tumoricidal effects of 4OHA in that system may reflect the induction of an intracellular response similar to that produced by pharmacological doses of estrogen.

MCF-7 cells provide a unique in vitro model for investigating the impact of potentially therapeutic aromatase inhibitors upon the hormonal regulation of human breast cancer cells. We anticipate that studies in this system will aid in the identification of clinically effective breast cancer therapies and provide an improved understanding of the biology of human breast cancer.

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References

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