ABSTRACT

Retinoic acid receptor (RAR) α has been shown to play a role in retinoid-induced growth inhibition of human breast cancer cell lines that express the estrogen receptor (ER). The dogma in the field has been that ER-positive breast cancer cell lines respond to retinoid treatment because they express RARα, whereas ER-negative breast cancer cell lines are refractory to retinoid treatment and have been thought to express little or no RARα. We set out to test several ER-negative breast cancer cell lines for expression of RARα protein and responsiveness to retinoids in growth inhibition assays. Of six ER-negative breast cancer cell lines that were tested, one (SK-BR-3) had high levels of RARα protein as measured by ligand-binding immunoprecipitation (55 fmol/mg protein) and also displayed sensitivity to growth inhibition by retinoids (9-cis-retinoic acid; EC50, 0.5 μM). Another ER-negative cell line, HS578T, also expressed RARα (23 fmol/mg) and was sensitive to retinoid-induced growth inhibition, albeit to a lesser extent than SK-BR-3 or T-47D cells. In contrast, the other ER-negative cell lines tested expressed low (<10 fmol/mg) or no detectable levels of RARα protein and also did not respond to retinoids in growth inhibition assays. A RARα agonist displayed 100 times greater potency than a RARγ agonist in growth inhibition of both T-47D and SK-BR-3 cells, suggesting RARα involvement in the process. Furthermore, a RARα antagonist completely abolished the growth inhibition induced by RAR agonists, implying that the activity of the agonists is exerted solely through RARα, not RARγ, which is also expressed in both cell lines. Additionally, although retinoid X receptor (RXR) compounds are weakly active in growth inhibition of the RARα-positive cell lines, they markedly increased the growth-inhibitory activity of RAR ligands. RXR compounds also potentiated the action of the antiestrogen 4-hydroxymefoxifen to inhibit the growth of T-47D cells. These findings have clinical ramifications in that patients with ER-negative tumors that are RARα positive may be candidates for retinoid therapy. Additionally, combinations of RXR ligands with RAR ligands (especially RARα agonists) and/or antiestrogens may have utility in the treatment of breast cancer.

INTRODUCTION

RARs and RXRs are members of the intracellular receptor superfamily that bind to their endogenous retinoid ligands with high affinity and specificity (1-4) and thereby regulate discrete sets of target genes (5). RARs bind to tRA and 9cRA with approximately equal affinity, whereas RXRs bind only to 9cRA. There are three known subtypes of each retinoid receptor subfamily, classified as α, β, and γ, which display specific patterns of expression throughout development and function in cell growth and differentiation processes (5-10).

RAR-active retinoids have been shown to inhibit the growth of certain breast cancer cells that express ER and may have potential as breast cancer therapeutic and/or preventive agents (11, 12). ER-positive breast cancer cells and tumors are dependent on estrogen for growth (13, 14) and exhibit sensitivity to growth inhibition by ER antagonists, such as tamoxifen (15). ER-positive breast cancer cell lines and tumors have been shown to express higher levels of RARα RNA (16-18) and protein (19) than do their ER-negative counterparts. Additionally, upon transfection of ER or RARα expression vectors into ER-negative, RARα-negative, retinoid-resistant breast cancer cells, retinoid sensitivity to growth inhibition is acquired by the cells (20, 21). These retinoid-resistant cells express RARγ; however, RARα is apparently required for them to display retinoid sensitivity. Therefore, RARα is thought to play a role in mediation of growth inhibition by retinoids in human breast cancer cells.

We have shown recently that, although ER-negative breast cancer tumor sections expressed lower RARα than did ER-positive tumor samples, as expected, some of the ER-negative tumors did express significant levels of RARα (19). Although the dogma in the field has been that ER-negative tumors or cells do not respond to retinoid treatment, we set out to test the hypothesis that a certain population of ER-negative breast cancer tumors do indeed express RARα and thereby might be responsive to treatment with retinoids. Clinically, ER-negative patients are generally relegated to chemotherapy and/or radiation treatment and have a worse prognosis than ER-positive patients. Therefore, a subgroup of ER-negative patients may exist that would represent a new pool of candidates for retinoid therapy. To test this hypothesis, a number of ER-negative breast cancer cell lines were assayed for RARα expression and responsiveness to retinoid-induced growth inhibition. Of six cell lines that were tested, two showed significant levels of RARα protein, determined by ligand-binding immunoprecipitation (22), and also demonstrated sensitivity to growth inhibition by retinoids. The other ER-negative cell lines tested expressed very low or nondetectable levels of RARα protein and were not growth inhibited by retinoids. These data are consistent with the hypothesis that RARα presence determines sensitivity of breast cancer cells to retinoids, whether or not they express ER.

Other groups have shown that retinoid growth-inhibitory effects in human breast cancer cells are elicited with RAR-specific compounds and not with RXR ligands (21). Using our RXR-selective ligands LG100268 (23) and LGD1069 (Targetin; Ref. 24), we observed weakly efficacious, but reproducible, growth inhibition of SK-BR-3 and T-47D cells. More importantly, the RXR-selective ligands increased the efficacy and potency of the RAR compounds upon administration in combination. Furthermore, RXR compounds potentiated the action of 4-OH-Tam to inhibit growth of the ER-positive cell line, T-47D. Therefore, RXR ligands may have utility in combination therapy of breast cancer.
MATERIALS AND METHODS

Ligands. 9cRA, LG100268 [6-(3,5,5,8-pentamethyl-5,6,7,8-tetrahydro-naphthalen-2-yl)cyclopropyl]nicotinic acid; Ref. 23], LGD 1096 [4-(1-(3,5,5,8-pentamethyl-5,6,7,8-tetrahydro-2-naphthyl)ethyl]benzoic acid; Ref. 24], ALRT1550 [(3'-hydroxy-4'-bromo-5',6',7',8'-tetrahydro-5',5',8',8'-tetramethylnaphthalen-2'-yl)carnamoyl]benzoic acid; Ref. 25], and an undefined carcinoma of the breast (BT-20) were maintained in Iscove’s MEM with 10% FBS. The cultures were maintained in a humidified atmosphere with 5% CO2 at 37°C. The cells were split at least twice a week by trypsinization and replated at dilutions between 1/2 and 1/8. Cell doubling times were as follows: for T-47D, MDA-MB-468, and MDA-MB-231, —24 h; for SK-BR-3, —36 h; for MDA-MB-435, —24 h; for Hs578T, BT-20, and MDA-MB-468, 1000 (8-day assays); and for Hs578T, BT-20, and MDA-MB-468, 1000 (8-day assays); for MDA-MB-435, 200—500 (6—8-day assays). Cells were harvested at 60—70% confluency, protein extracts were prepared as described previously (4, 22), and ligand binding-immunoprecipitation assays were carried out as detailed elsewhere (22). All antibodies used in the assays are subtype selective as shown previously; i.e., each antiretinoid receptor antibody precipitates only its respective receptor and not the other five subtypes, as demonstrated with recombinantly expressed receptor-containing extracts (22). This assay was also validated as an accurate and specific method to detect and quantitate endogenous levels of retinoid receptors in cell and tissue extracts (22). Briefly, extracts (500 µg/tube) were incubated with 10 nM [3H]RA or 20 nM [3H]9cRA with or without a 200-fold molar excess of the respective unlabeled retinoid in binding buffer containing 0.4 mM KCl, 10 mM Tris (pH 7.5), and 0.5% 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid detergent. Ligand-extract incubation took place in glass tubes for 16 h at 4°C. The mixture was then transferred to siliconized microfuge tubes, purified subtype-selective retinoid receptor antibodies (22) were added, and incubation was continued for 4 h at 4°C. Protein A-Sepharose (Pharmacia) was added for 45 min at 4°C, rotating end-over-end. Protein A-Sepharose pellets were washed twice alternatively with high salt binding buffer (see above) and with the same buffer containing 0.15 M KCl and then quantitated for tritium by scintillation counting. Ligand-binding Immunoprecipitation Assays. Cells were harvested at 60—70% confluency, protein extracts were prepared as described previously (4, 22), and ligand binding-immunoprecipitation assays were carried out as detailed elsewhere (22). All antibodies used in the assays are subtype selective as shown previously; i.e., each antiretinoid receptor antibody precipitates only its respective receptor and not the other five subtypes, as demonstrated with recombinantly expressed receptor-containing extracts (22). This assay was also validated as an accurate and specific method to detect and quantitate endogenous levels of retinoid receptors in cell and tissue extracts (22). Briefly, extracts (500 µg/tube) were incubated with 10 nM [3H]RA or 20 nM [3H]9cRA with or without a 200-fold molar excess of the respective unlabeled retinoid in binding buffer containing 0.4 mM KCl, 10 mM Tris (pH 7.5), and 0.5% 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid detergent. Ligand-extract incubation took place in glass tubes for 16 h at 4°C. The mixture was then transferred to siliconized microfuge tubes, purified subtype-selective retinoid receptor antibodies (22) were added, and incubation was continued for 4 h at 4°C. Protein A-Sepharose (Pharmacia) was added for 45 min at 4°C, rotating end-over-end. Protein A-Sepharose pellets were washed twice alternatively with high salt binding buffer (see above) and with the same buffer containing 0.15 M KCl and then quantitated for tritium by scintillation counting.

Growth Inhibition Assays and Analysis. Cells were plated in 96-well flat-bottomed microtiter plates (CoStar) in 50 µl of serum-containing medium/well. All cell growth assays were carried out in medium containing 7.5% FBS except for assays with T-47D and Hs578T cells, which were performed in 10% FBS-containing medium. Cell plating numbers (cells/well) were as follows: for SK-BR-3 and T-47D, 5000 (1-day assays), 4000 (2-day assays), 3000 (3-day assays), and 2000 (4- and 6-day assays); for MDA-MB-435, 200—500 (6—8-day assays); and for Hs578T, BT-20, and MDA-MB-468, 1000 (8-day assays). Cell plating number was determined by the growth rate of each cell line and duration of the assay. Cells treated with vehicle were ~80% confluent by the end of each assay. Cells were allowed to adhere for 24 h prior to compound addition as follows. Ten-fold serial dilutions of compounds were performed, with changing of pipette tips between each dilution. Diluted compounds were then added to the cells (50 µl/well) to final concentrations of 1 × 10—3 M to 1 × 10—11 M (1% ethanol), in triplicate. Cells and ligands were incubated at 37°C for 3—8 days (see individual figure legends) with replenishment of ligands in fresh medium every 3 days. BrdUrd incorporation was analyzed as a measure of DNA replication (Cell Proliferation ELISA kit; Boehringer Mannheim), and absorbance was measured at 450 nm. Cell number and viability were determined by the use of a MTS cell proliferation assay (Promega). The tetrazolium compound is bioreduced by live cells to a soluble formazan that absorbs at 490 nm and is directly proportional to the number of living cells in the sample. Assay duration was determined by cell line growth rate and the assay type. BrdUrd assays yielded optimal effects at 3—5 days, whereas the MTS assays did not show marked effects until 5—8 days, depending on the cell line.

One row of wells contained medium only, and these absorbance values were averaged as background and then subtracted from absorbance values from each individual well containing cells. The average values were then determined for each triplicate and plotted versus ligand concentration. The average vehicle (1% ethanol) value was determined, less the background average. This value is displayed on graphs (see Figs. 1—9) as a single point at 0 M and is denoted as a solid diamond. Efficacy (% growth inhibition) was determined as 1 — (A490 nm or A580 nm at maximal response with compound/A490 nm or A580 nm of average vehicle) × 100. Potency (EC50) was determined as the concentration of compound that elicited a maximal-halve response.

RESULTS

Quantitation of Retinoid Receptor Levels in Human Breast Cancer Cell Lines. A number of breast cancer cultured cell lines were analyzed for RAR and RXR protein levels by the use of a previously validated ligand-binding immunoprecipitation assay using subtype-selective antiretinoid receptor antibodies (23). ER status of the cells was confirmed by the use of an estrogen-ER binding assay. T-47D, an ER-positive breast cancer cell line, expressed both RARα and RARγ at approximately equal levels (~35 fmol/mg) and did not have detectable levels of RARβ protein (Table 1). SK-BR-3 and Hs578T, ER-negative cell lines, expressed significant levels of RARα protein (~55 fmol/mg and ~23 fmol/mg, respectively). SK-BR-3 cells also expressed RARγ, but RARβ protein was undetectable using our method. Three ER-negative cell lines listed in Table 1 (MDA-MB-468, BT-20, and MDA-MB-231) expressed RARγ but had low or undetectable levels of RARα and RARβ proteins (~<10 fmol/mg), whereas MDA-MB-435 had low RARα but significant levels of RARβ and RARγ. The individual cell lines were then tested for responsiveness to retinoid-induced growth inhibition to determine whether there was a correlation between retinoid sensitivity and expression of RARα protein.

Growth Inhibition of Breast Cancer Cells by RAR-Active Ligands. Cells were grown and assessed for growth inhibition by BrdUrd incorporation, a measure of DNA replication, and by use of a MTS assay, a measure of cell viability, as described in “Materials and Methods.” Optimal effects on DNA replication (BrdUrd assay) were

<table>
<thead>
<tr>
<th>Table 1</th>
<th>ER status and retinoid receptor protein levels of various breast cancer cell lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell line</td>
<td>ER</td>
</tr>
<tr>
<td>T-47D</td>
<td>+</td>
</tr>
<tr>
<td>SK-BR-3</td>
<td></td>
</tr>
<tr>
<td>Hs578T</td>
<td></td>
</tr>
<tr>
<td>MDA-MB-435</td>
<td></td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td></td>
</tr>
<tr>
<td>BT-20</td>
<td></td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td></td>
</tr>
</tbody>
</table>

* Femtomoles of receptor per mg total soluble protein as determined by ligand-binding immunoprecipitation (22). Values were determined from at least two individual experiments performed in duplicate.

** BLD, below limits of detection.

* ND, not determined.
Table 2  

<table>
<thead>
<tr>
<th>Cell</th>
<th>BrdU&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MTS&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK-BR-3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>NE&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>NE</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>NE</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>77</td>
</tr>
<tr>
<td>8</td>
<td>ND</td>
<td>3</td>
</tr>
<tr>
<td>T-47D</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>ND</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>ND</td>
<td>89</td>
</tr>
</tbody>
</table>

<sup>a</sup>See "Materials and Methods" for calculation formulas for EC<sub>50</sub> (potency) and percentage growth inhibition (efficacy) values. Each value represents determination from a full dose range (10<sup>-11</sup> to 10<sup>-5</sup> M) with TTNPB performed in triplicate.

<sup>b</sup>NE, no effect.

<sup>d</sup>ND, not determined.

observed typically between 3 and 5 days, depending on the cell line (see illustrative example of TTNPB in Table 2; data not shown). Cell number effects (as measured by the MTS assay) were apparent at later time points (optimal at 5–8 days), because they follow the DNA replication decrease (Table 2; data not shown). Time courses of growth inhibition were performed with each cell line with TTNPB (Table 2), 9cRA (data not shown), and ALRT1550 (data not shown) for each assay. Potencies of the compounds were virtually unchanged over time; however, efficacy (percentage of growth inhibition) increased (see Table 2). Optimal time points were chosen for each assay (earliest or most convenient day that significant efficacy was observed with the compounds tested) and are shown in the figures. Fig. 1 shows the results of BrdUrd assay (Fig. 1A) and MTS assay (Fig. 1B) after treatment of ER-positive T-47D cells for 3 (Fig. 1A) and 6 (Fig. 1B) days with various RAR-active compounds: ALRT1550 (25) and TTNPB (26), both synthetic, potent compounds that bind and activate the RARs but not the RXRs, and 9cRA, a naturally occurring RAR- and RXR-active compound (Refs. 1–4; Table 3). ALRT1550 was the most potent growth inhibitor of these cells (EC<sub>50</sub> of 0.1–0.3 nM). TTNPB displayed an EC<sub>50</sub> of ~6 nM in T-47D cells, and efficacy was reproducibly less than that elicited with ALRT1550. 9cRA was the least potent of these three compounds but was fully efficacious in inhibition of DNA replication and growth of these cells.

In ER-negative SK-BR-3 cells, 9cRA and TTNPB displayed very similar profiles of growth inhibition and were equipotent (EC<sub>50</sub> of ~3 nM) in a 6-day MTS assay (Fig. 2A) and in 3-day BrdUrd assays (Figs. 4B and 5B). ALRT1550 was also more potent than these ligands in SK-BR-3 cells, with EC<sub>50</sub> values of ~0.3 nM in the MTS assay (Fig. 2A) and in 3-day BrdUrd assays (data not shown). The ER-negative Hs578T cell line, which showed intermediate levels of RARα protein (Table 1), was also responsive to retinoid-induced growth inhibition, as assessed by both a 6-day BrdUrd assay (data not shown) and an 8-day MTS assay (Fig. 2B). This inhibition was not as robust as that observed with the T-47D and SK-BR-3 cells (which expressed higher levels of RARα), as evidenced by the longer duration of the assays to achieve comparable efficacies (compare Figs. 1 and 2A with Fig. 2B).

In contrast to the RARα-positive T-47D, SK-BR-3, and Hs578T cells, the cell lines that had low or undetectable levels of RARα protein (Table 1) were unresponsive to retinoids in both BrdUrd and MTS growth inhibition assays performed at various time points from 2 to 10 days (Fig. 3; data not shown). MDA-MB-231 cells also did not respond to ALRT1550 or TTNPB (data not shown). Interestingly, whereas all of these nonresponsive cells expressed RARγ, one of them expressed RARβ (MDA-MB-435) but also was not growth inhibited by retinoids. Therefore, of the seven breast cancer cell lines that were tested, only the RARα-positive cells were responsive to growth-inhibitory effects of RAR-active retinoids.

**Growth Inhibition of Breast Cancer Cells by RAR Subtype-selective Compounds.** We next tested the effects of subtype-selective ligands in growth inhibition assays to determine which retinoid receptor(s) was responsible for the activity in the two most retinoid-responsive cell lines discussed above. Fig. 4 shows the results from BrdUrd assays of both T-47D and SK-BR-3 cells treated with 9cRA; AGN193835, an RARα-selective agonist (Ref. 30; see Table 3); or CDE666, a RARγ-selective agonist (Ref. 28; see Table 3). The RARα-selective ligand, AGN193835, was more potent than 9cRA in T-47D cells (Fig. 4A; EC<sub>50</sub> of ~2 nM) and was equipotent to 9cRA in SK-BR-3 cells (Fig. 4B; EC<sub>50</sub> of ~1 nM). ALRT1550 was also more potent than 9cRA in SK-BR-3 cells (Fig. 4A; EC<sub>50</sub> of ~2 nM), but was equipotent to 9cRA in T-47D cells (Fig. 4B; EC<sub>50</sub> of ~1 nM).

**Fig. 1.** RAR-active retinoids inhibit DNA replication and growth of ER-positive, RARα-positive breast cancer cells. T-47D cells were grown and plated at 3000 (A) or 2000 (B) cells/well in 96-well plates, as described in "Materials and Methods." ALRT1550, 9cRA, and TTNPB were added in ethanol vehicle in medium (see "Materials and Methods") at seven concentrations between 10<sup>-11</sup> and 10<sup>-5</sup> M. After 3 days, a BrdUrd incorporation ELISA assay was performed (A). The colorimetric reaction was stopped, and absorbance was measured at 450 nm. After 6 days, a MTS cell viability assay (B) was performed (compounds were replenished in fresh medium after 3 days). The enzymatic reaction product was measured at 490 nm. •, vehicle average.
cells (Fig. 4B; EC_{50}; ~2 nM). The RARγ agonist was least potent in both cell lines (EC_{50}; ~300 nM in T-47D and ~200 nM in SK-BR-3). This RARγ ligand, CD666, has been tested by us in a reporter-based cotransfection assay (using TRE-pal in CV-1 cells; see Table 3 and Refs. 4 and 5) for relative activity via each RAR subtype to confirm its selectivity profile. CD666 was most potent and efficacious via RARγ (EC_{50}; ~9 nM) and displayed some activity on RARβ (EC_{50}; ~400 nM) and weak activity on RARα (EC_{50}; ~1–2 μM). Therefore, although CD666 is selective for RARγ, it retains residual activity via RARα, which may account for its activity at micromolar concentrations (see Fig. 4). To determine whether this was the case, RAR-specific antagonists were tested for their ability to block the effects of the RAR agonists.

Blocking of Retinoid-induced Growth Inhibition of Breast Cancer Cells by RAR-Selective Antagonists. A RAR-selective antagonist that acts at each of the three RAR subtypes, AGN193109 (Ref. 29; see Table 3), and a RARα-selective antagonist, Ro 41-5253 (Ref. 27; see Table 3), were tested in BrdUrd assays in both T-47D and SK-BR-3 cells for their ability to inhibit the effects of TTNPB, an RAR-pan-agonist in eliciting growth effects on the cells. Fig. 5 shows the effect of AGN193109 to block inhibition of DNA replication by TTNPB in T-47D (Fig. 5A) and SK-BR-3 (Fig. 5B) cells. AGN193109 alone had no effect on growth inhibition of the cells but completely blocked activity of TTNPB (held constant at 20 nM (Fig. 5A) and 5 nM (Fig. 5B)) in a dose-dependent manner, with IC_{50} of 5–10 nM. The RARα-selective antagonist, Ro 41-5253, also showed no effect on its own in DNA replication inhibition, and completely blocked the effects of TTNPB in both T-47D and SK-BR-3 cell BrdUrd assays (Fig. 6). Ro 41-5253 displayed potencies of ~50–100 nM to inhibit the effects of TTNPB in these cells. This reduced potency in comparison to AGN193109 is most likely due to the fact that Ro 41-5253 is a less potent binder of RARα than AGN193109 (data not shown) and is less potent than AGN193109 as an antagonist in reporter-based assays for RAR activity (see Table 3). More important than the potency of Ro 41-5253 is its ability to achieve 100% efficacy in blockage of the effect of TTNPB in growth inhibition of these cells, implying that TTNPB requires RARα to elicit its effects.

RXR Ligands Potentiate the Activity of RAR Ligands to Inhibit the Growth of Breast Cancer Cells. Recently, it was shown that RAR ligands alone were not able to differentiate embryonal carcinoma cells but that they potentiated the effects of RAR ligands (32). Therefore, whereas others have shown that RAR ligands were inert in eliciting growth inhibition in breast cancer cells (21), we tested the possibility that RAR ligands might modulate the effects of RAR ligands to inhibit the growth of breast cancer cells. Two RXR-selective ligands were used for this purpose: LG100268 (23, 33), a highly specific RXR compound, and LGD1069 (24), a RXR-selective ligand that is currently being tested in the clinic for various diseases, including cancer. LGD1069 is less potent than LG100268 in RXR binding and cotransfection experiments (Refs. 23 and 24; see Table 3) and displays some RXR cross-reactivity at high ligand concentrations (Table 3). Fig. 7 shows that LG100268 alone elicited a weakly efficacious, but reproducible, effect in growth inhibition of T-47D and SK-BR-3 cells. LG100268 in combination with a constant concentration of TTNPB (5 nM (Fig. 7A) or 2 nM (Fig. 7B)) yielded increased inhibition of BrdUrd incorporation versus either ligand alone. This effect was also observed with the other RXR compound, LGD1069 (Fig. 8). These results indicate that RXR ligands work in concert with RAR ligands, at least in these two breast cancer cell lines, to yield increased cell growth inhibition.
ROLE OF RARα IN GROWTH INHIBITION OF BREAST CANCER

We were surprised to observe the effects of RXR-selective ligands alone in growth inhibition of the breast cancer cells tested herein (Figs. 7 and 8). In light of their activity, we tested whether they would be able to potentiate the effects of antiestrogens, which has been demonstrated previously for RAR-active ligands (34). Fig. 9 shows that LGD1069 (Fig. 9A) and ALRT1550 (Fig. 9B) each potentiated the effects of 4-OH-Tam in DNA replication assays in T-47D cells. 4-OH-Tam at a constant concentration of 10 nM yields ~50% response in BrdUrd assays in T-47D cells. An increasing dose of LGD1069 or ALRT1550 (Fig. 9) or 9cRA or LG100268 (data not shown) augments the response in the assay. These retinoids also potentiated the effect of another antiestrogen, ICI164384, in growth inhibition of T-47D cells (data not shown). The potential use of RXR ligands in the treatment of cancer in combination with antiestrogens may be quite promising, given that RXR ligands have not displayed classical retinoid toxicity in humans (35).

DISCUSSION

ER-positive breast cancer cell lines are known to express RARα RNA (16–18) and protein (22), and their growth is inhibited by retinoids. Several ER-negative lines have been shown to be refractory to retinoid treatment and to have low or no RARα RNA. Therefore, the dogma in the field has been that ER-negative breast cancer cells do not respond to retinoids, because they do not have RARα. Recently, we showed that a number of ER-negative breast tumor samples were positive for RARα, as assessed by immunohistochemistry (19). We set out to test the hypothesis that some ER-negative breast cancer cells may express RARα and that its presence might correlate with the ability of retinoids to inhibit the growth of those cells, as they do for ER-positive cells. To do this, a number of ER-negative breast cancer cell lines were assayed for the presence of functional (ligand binding-capable) RARα protein by use of a ligand-binding immunoprecipitation assay (22) and for their response to retinoids in growth inhibition assays.

Table 1 and Fig. 2 show that of the six ER-negative breast cancer cell lines that were tested, one (SK-BR-3) expressed relatively high levels or RARα protein and was also very sensitive to growth inhibition by retinoids. Another ER-negative cell line, Hs578T, also expressed RARα, but at a lower level than SK-BR-3 cells (Table 1), and its growth was also inhibited by retinoids (Fig. 2B), albeit to a lesser extent than SK-BR-3 cells (Fig. 2A). Hs578T cells have been reported to be retinoid resistant (17); however, earlier work showed that these cells responded to retinoids in growth inhibition assays (36). Data shown here (Fig. 2) were produced from fresh stocks of cells from American Type Culture Collection, and conflicting reports may be due to cells becoming retinoid resistant with increasing passage. Interestingly, all of the cell lines tested expressed RARγ protein, and one also expressed high levels of RARβ (MDA-MB-435), but expression of these receptors did not correlate with the ability of the cells to respond to retinoids. Therefore, RARα expression does not depend on
ROLE OF RARα IN GROWTH INHIBITION OF BREAST CANCER

their growth is not inhibited by retinoids (Table 1; Fig. 3). Another group has shown recently that RARα expression in breast lesion specimens correlated with proliferative activity and was independent of ER presence (39). Therefore, evidence is mounting that there may be a subset of ER-negative breast cancer patients whose tumors are RARα-positive and, hence, may be responsive to retinoid therapy.

The advent of subtype-selective retinoids has allowed the testing of these compounds in various settings to determine the receptor component that is involved in a cellular process of interest. AGN193835 is a RARα-selective ligand as determined by receptor binding specificity and cotransfection-cotransactivation assays using the three RARs and three RXRs (30). This compound was a potent growth inhibitor of both RARα-expressing breast cancer cell lines that were

ER presence in the cell, even though estrogen can up-regulate RARα RNA (37) and protein (22) in ER-positive breast cancer cells. Furthermore, the work herein shows that if RARα is expressed, the cell has the ability to respond to retinoids regardless of ER presence.

Recently, other workers have shown that SK-BR-3 cells have ~2-fold more RARα RNA than other ER-negative cell lines and respond to 6-day treatment with 1 μM of various retinoids by cell number assays (38). Herein, we have observed ~5–10-fold more RARα protein in SK-BR-3 cells versus the ER-negative lines (Table 1) and show that they are exquisitely sensitive to retinoids at nanomolar concentrations in DNA incorporation and cell viability assays (Figs. 2 and 4). These workers also showed that MDA-MB-435 cells expressed RARα RNA at comparable levels to SK-BR-3 cells (38); in contrast, we found that MDA-MB-435 cells have low levels of RARα protein (approximately equal to other ER-negative cell lines), and

Fig. 4. A RARα-selective ligand is more potent than a RARβ,γ compound in growth inhibition of breast cancer cells. T-47D (A) and SK-BR-3 (B) cells were grown and plated as described in Fig. 1 and in "Materials and Methods." 9cRA, AGN193835 (RARα-selective), and CD666 (RARγ-selective) were added in ethanol vehicle in medium (see "Materials and Methods") at seven concentrations between 10⁻⁸ and 10⁻¹¹ M. After 3 days, BrdUrd incorporation ELISA assays were performed. The colorimetric reaction was stopped, and absorbance was measured at 450 nm. •, vehicle average.

Fig. 5. A RAR-pan-antagonist blocks TTNPB-induced growth inhibition of breast cancer cells. T-47D (A) and SK-BR-3 (B) cells were grown and plated as described in Fig. 1 and in "Materials and Methods." TTNPB or AGN193109 (RAR-pan-antagonist) were added in ethanol vehicle in medium (see "Materials and Methods") at six concentrations between 10⁻⁶ and 10⁻¹¹ M. TTNPB was held constant at 2 (A) and 5 nM (B), and AGN193109 was added in combination with TTNPB in a dose-dependent manner, as above. After 3 days, BrdUrd incorporation ELISA assays were performed. The colorimetric reaction was stopped, and absorbance was measured at 450 nm. •, vehicle average.

2647

Downloaded from cancerres.aacrjournals.org on July 29, 2017. © 1997 American Association for Cancer Research.
ROLE OF RARα IN GROWTH INHIBITION OF BREAST CANCER

cancer cell growth inhibition assays and have been reported to have no effect (21). However, in light of a recent report that showed that although RXR ligands alone had no effect in differentiation of embryonal carcinoma cells, there was synergy observed between RAR and RXR compounds (32), we tested two of our RXR ligands alone and in combination with RAR compounds in breast cancer cell growth inhibition assays. Figs. 7 and 8 show that each of two RXR-selective ligands, LG100268 (23) and LGD1069 (Targretin; Ref. 24), were able to weakly, but reproducibly, inhibit the growth of both T-47D and SK-BR-3 cells in 3-day BrdUrd assays. Also, both compounds potentiated the effects of RAR ligands, yielding increased efficacy in growth inhibition at a half-maximal dose of RAR ligand. The activity that is observed with RXR ligands alone may be due to potentiation by

Fig. 6. A RARα-selective antagonist blocks TTNPB-induced growth inhibition of breast cancer cells. T-47D (A) and SK-BR-3 (B) cells were grown and plated as described in Fig. 1 and in “Materials and Methods.” TTNPB and Ro 41-5253 (RARα-selective antagonist) were added in ethanol vehicle in medium (see “Materials and Methods”) at six concentrations between 10⁻⁶ and 10⁻¹¹ M. Additionally, TTNPB was held constant at 2 nM (A) and 5 nM (B), and Ro 41-5253 was added in combination with TTNPB in a dose-dependent manner, as above. After 3 days, BrdUrd incorporation ELISA assays were performed. The colorimetric reaction was stopped, and absorbance was measured at 450 nm. •, vehicle average.

tested: ER-positive T-47D cells and ER-negative SK-BR-3 cells (Fig. 4). A compound that is selective for RARγ, with very weak activity via RARα, CD666 (28), is 100 times less potent in growth inhibition of these two cells (Fig. 4). Furthermore, a RARα-selective antagonist, Ro 41-5253 (27), was able to completely block the effects of RAR agonists, such as TTNPB, in growth inhibition of both cell lines, implying that the activity of the agonists is through RARα (Fig. 6). Clinically, RARα compounds may be attractive drugs for breast cancer, in that much of the toxicity observed with retinoids has been shown to be due to activity through RARγ (40, 41).

RXR-selective compounds have been tested previously in breast
ROLE OF RARα IN GROWTH INHIBITION OF BREAST CANCER

The activity of RXR ligands in breast cancer cell growth inhibition prompted us to study their potential in combination with antiestrogens. The antiestrogen tamoxifen is currently the drug of choice in treatment of patients with ER-positive breast cancer. Antiestrogens have been shown to have increased activity in growth inhibition of ER-positive breast cancer cells in the presence of RAR-active retinoids (34). Fig. 9 shows that the RXR-selective ligand LGD1069 potentiates the effect on a constant dose of 10 nM 4-OH-Tam in a T-47D cell growth assay. LG100268 displays the same behavior (data not shown), and the RAR-active compound ALRT1550 is also more effective in combination with 4-OH-Tam than alone, as expected (Fig. 9). RXR compounds have also been shown to have additive effects with RAR compounds in other cancers (42–44). This novel activity of RXR ligands in breast cancer is interesting in that the RXR compound, LGD1069, has shown markedly

---

**Fig. 8.** The RXR-active ligand LGD1069 potentiates the effect of a RAR ligand to inhibit the growth of breast cancer cells. T-47D (A) and SK-BR-3 (B) cells were grown and plated as described in Fig. 1 and in “Materials and Methods.” TTNPB and LGD1069 (RXR-selective agonist) were added in ethanol vehicle in medium (see “Materials and Methods”) at six concentrations between 10^-5 and 10^-11 M. Additionally, TTNPB was held constant at 5 (A) and 2 nM (B), and LGD1069 was added in combination with TTNPB in a dose-dependent manner, as above. After 3 days, BrdUrd incorporation ELISA assays were performed. The colorimetric reaction was stopped, and absorbance was measured at 450 nm. •, vehicle average.

---

**Fig. 9.** Both RXR ligands and RAR ligands potentiate the effects of an antiestrogen to inhibit the growth of breast cancer cells. T-47D cells were grown and plated as described in Fig. 1 and in “Materials and Methods.” LGD1069 (A; RXR-selective agonist), ALRT1550 (B; RAR-pan-agonist), and 4-OH-Tam (A and B; antiestrogen) were added in ethanol vehicle in medium (see “Materials and Methods”) at seven concentrations between 10^-5 and 10^-11 M. Additionally, 4-OH-Tam was held constant at 10 nM, and LGD1069 (A) and ALRT1550 (B) were added in combination with 4-OH-Tam in a dose-dependent manner as above. After 3 days, BrdUrd incorporation ELISA assays were performed. The colorimetric reaction was stopped, and absorbance was measured at 450 nm. •, vehicle average.

---

serum factors and/or ligands present in serum that is present in the assays (5–7.5% FBS, depending on cell line). These assay conditions were thought to be appropriate in that ultimately we hope to predict usefulness in the clinic, where human patients are the targets. Both T-47D and SK-BR-3 cells were found to express significant levels of RARα protein, as did other ER-negative cell lines that did not respond to retinoids (Table 1). Therefore, responsiveness of breast cancer cells to RXR compounds, either alone or in combination with a RAR compound, seems to depend on RARα expression, because RXR expression alone is not sufficient. The active unit of at least one step in the retinoid-induced growth inhibition pathway in breast cancer cells may, hence, be a RARα:RXR heterodimer.
less toxicity than pan-RAR-active retinoids in humans (35) and may be useful as a therapeutic in combination with other treatments. In breast cancer, it is tempting to speculate that a RARα drug in combination with an RXR ligand with (ER positive) or without (ER negative) tamoxifen may produce a superior therapeutic index than either tamoxifen or chemotherapy alone.

ACKNOWLEDGMENTS

We thank M. Grottidis, W. Lamph, and M. Boehm for helpful discussions and M. Grottidis for provision of the insulin-independent T-47D cell line. We thank the synthesis of AGN193109, M. Boehm and L. Zhang for ALR15150 and TTNPB, S. Jeong and L. Farmer for CD664, and L. Dardashti and S. Canan-Koch for RI 41-5253.

REFERENCES


Retinoic Acid Receptor α Expression Correlates with Retinoid-induced Growth Inhibition of Human Breast Cancer Cells Regardless of Estrogen Receptor Status

Patrick Fitzgerald, Min Teng, Roshantha A. S. Chandraratna, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/57/13/2642