Inhibition of Breast Cancer Cell Growth in Vitro by a Tyrosine Kinase Inhibitor

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Introduction

It has been proposed that breast cancer growth is regulated in part via the endogenous synthesis and secretion of polypeptide growth factors by the tumor itself, as well as by growth factors produced at distant sites (1, 2). These growth factors bind to specific membrane receptors and activate the receptor tyrosine kinase (TK) activity, which is essential for signal transduction and biological function (3-5). The crucial role of receptor TK activation in mediating the effects of several different polypeptide growth factors identifies these proteins as potential targets for selective chemotherapy.

Tyrosinostatins are a group of low molecular weight synthetic compounds that inhibit tyrosine kinase activity (6-8). RG-13022 is a new tyrosine kinase inhibitor that has been reported to have even more selectivity for the EGF receptor in cell-free assays than do the prototype compounds in cell-free autophosphorylation assays, the 50% inhibitory concentration of RG-13022 for the EGF receptor is 1 to 3 \( \mu \text{M} \), compared to a 50% inhibitory concentration of 25 \( \mu \text{M} \) for the platelet-derived growth factor receptor. This inhibitor is overexpressed in some breast cancers, and its presence is associated with a more aggressive clinical course, suggesting that it has an important growth-regulatory function in breast cancer (9, 10). Blockade of this receptor inhibits the growth of human breast cancer cells (11). In the present study, we examined the effects of RG-13022 on a panel of human breast cancer cell lines that are mitogenically responsive to a variety of different growth factors, including EGF. We have found that RG-13022 is a potent inhibitor of breast cancer cell proliferation and that it blocks the stimulatory effects of several different polypeptide growth factors, as well as the effects of estrogen.

Materials and Methods

Cells and Cell Culture. The MDA-231 and ZR75-1 cell lines were obtained from the American Type Culture Collection (Rockville, MD). The MCF-7 line was supplied by Dr. Marc Lippman (Lombardi Cancer Research Center, Washington DC), the BT-20 line by Dr. William L. McGuire (University of Texas Health Science Center at San Antonio, San Antonio, TX), and the T47D line by Dr. Nancy Davidson (Johns Hopkins Medical School, Baltimore, MD). All breast cancer cell lines were cultured in IMEM (GIBCO Laboratories, Grand Island, NY), supplemented with 5 to 10% FCS (GIBCO) and 10 nM insulin (Eli Lilly Research Laboratories, Indianapolis, IN), as described previously (11). CHO cells were cultured in alpha minimum essential medium (GIBCO) supplemented with 10% FCS. All cell lines were routinely tested for Mycoplasma contamination and were not infected.

Growth Factors and Tyrosine Kinase Inhibitor. Epidermal growth factor (EGF) derived from mouse submaxillary gland was purchased from Collaborative Research Laboratories (Lexington, MA). Recombinant human TGF-alpha was provided by Dr. R. Derynck (Genentech Inc., San Francisco, CA). Human insulin was purchased from Eli Lilly Research Laboratories. Insulin-like growth factor I was purchased from Collaborative Research Laboratories. Insulin-like growth factor II was supplied by Dr. C. H. Li (University of California at San Francisco), and 17beta-estradiol was purchased from Sigma Chemical Co. (St. Louis, MO). The tyrosine kinase inhibitor used in this study, RG-13022 (Fig. 1), was synthesized and kindly provided by Dr. P. Persons and Dr. A. Spada of Rhone-Poulenet Rorer. Stock solutions were made in DMSO and diluted to appropriate concentrations in culture medium prior to addition to the cells. An equivalent dilution of DMSO (0.1%) without the inhibitor served as a control.

DNA Synthesis. Cells were plated in 24-well tissue culture dishes (Corning), at a density of 1 x 10^3 cells/well, in their regular growth medium. After 24 h, the seeding medium was removed, the cells were washed twice with PBS, and the cells were incubated in serum-free and phenol red-free IMEM (1 ml/well). Twenty-four h later, growth factor and/or RG-13022 were added. After 16 h, 0.25 \( \mu \text{Ci} \) of [3H]thymidine (82.3 Ci/mmol; NEN Products, Boston, MA), in a volume of 25 \( \mu \text{l} \), was added to each well for a 1-h pulse. Cells were harvested and the rate of DNA synthesis was estimated by measuring the acid-precipitable radioactivity, as described previously (12).

Radioactivity was quantified in a Beckmann LS 7000 liquid scintillation counter with an efficiency of 64%.

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4 The abbreviations used are: TK, tyrosine kinase; EGF, epidermal growth factor; IGF-I, insulin-like growth factor I; IGF-II, insulin-like growth factor II; IGF-I, insulin-like growth factor II; MEM, Iscove's minimum essential medium; FCS, fetal calf serum; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; TGF-a, transforming growth factor alpha; DMSO, dimethyl sulfoxide; ER, estrogen receptor.

5 A. Zilberstein, unpublished observations.
Inhibition of EGF Receptor Autophosphorylation by RG-13022. It has previously been shown that tyrphostins are potent inhibitors of the tyrosine kinase activity of the EGF receptor in a variety of target cells in vitro (7, 8, 16). To confirm that these compounds can block EGF receptor autophosphorylation in breast cancer cells, we examined the effect of RG-13022 on MDA-231 breast cancer cells, which contain relatively large numbers of EGF receptors (17). After a 5-min exposure of these cells to EGF, Western analysis using antiphosphotyrosine antibodies demonstrated significant inhibition of EGF-stimulated autophosphorylation of a M, 170,000 band, representing the EGF receptor (Fig. 2). Tyrosine phosphorylation of the EGF receptor was inhibited in a dose-dependent manner (Fig. 3); half-maximal inhibition was observed at a concentration of about 2 µM.

Inhibition of EGF-stimulated Breast Cancer Cell Proliferation by RG-13022. To investigate the effects of RG-13022 on EGF-stimulated cell proliferation, we examined its effects on MCF-7 cell number after 5 days in culture (Fig. 4). EGF induced a 2–3-fold increase in cell number, and this effect was totally blocked by 5 µM RG-13022. At a concentration of 10 µM, RG-13022 inhibited cell growth significantly below controls grown in the absence of EGF, suggesting that, at this concentration, RG-13022 might inhibit other growth-regulatory processes, in

**RESULTS**

The results are shown in Fig. 4. EGF (10 nM) induced a 2–3-fold increase in cell number, and this effect was totally blocked by 5 µM RG-13022. At a concentration of 10 µM, RG-13022 inhibited cell growth significantly below controls grown in the absence of EGF, suggesting that, at this concentration, RG-13022 might inhibit other growth-regulatory processes, in

**Fig. 2.** Effect of RG-13022 on EGF-stimulated autophosphorylation of the EGF receptor. MDA-231 cells were grown in IMEM, with serum, to near-confluence. The seeding medium was removed, cells were washed twice with PBS, and the medium was replaced with phenol red-free and serum-free IMEM. Eight h later RG-13022 or DMSO (controls) was added to the cultures. After an additional 16 h, EGF (10 nM) was added for 5 min, and EGF receptor phosphorylation was evaluated by Western blot analysis, as described in “Materials and Methods.”

**Fig. 3.** Western blot analysis of autophosphorylation of the EGF receptor. MDA-231 cells were grown in IMEM, with serum, to near-confluence. The seeding medium was removed, cells were washed twice with cold PBS. The reaction was stopped by adding 2 ml of ice-cold lysis buffer [50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, 2 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 12.5 µg/ml leupeptin, 0.5% aprotinin, 1 mM phenylmethylsulfonylfluoride, 1 mM sodium vanadate, 4 mM sodium p-nitrophenylphosphate, 100 mM sodium fluoride]. The mixture was shaken, liquid nitrogen was poured directly into the lysis buffer to freeze the cells quickly, and the lysate was then thawed on ice. The samples were centrifuged at 14,000 × g for 30 min, and the supernatant was used for immunoprecipitation. The protein concentration was determined in the supernatant by the bicinchoninic acid method (13) and was then normalized in all the samples. The EGF receptor immunoprecipitation was done by using EGF receptor antibody 528 (provided by Dr. John Mendelsohn, Memorial Sloan-Kettering Cancer Center, New York, NY) conjugated with rabbit anti-mouse immunoglobulin and Protein A. The proteins were then solubilized in Laemmli buffer with 100 µM dithiothreitol, with pyronin to give the buffer sufficient color for use as a tracking dye, boiled for 10 min, and separated in 7.5% polyacrylamide gels, under denaturing and reducing conditions, as described by Laemmli (14). Resolved proteins were transblotted to 0.45-μm nitrocellulose membranes (Schleicher and Schuell) at 200 mA for 16 h, at 4°C, by the method of Towbin et al. (15). After blocking with 3% bovine serum albumin (fraction V; Boehringer Mannheim) for 1 h, the blots were incubated with antiphosphotyrosine antibody PY20 (1:3000; ICN Biochemicals, Cleveland, OH) overnight at 4°C. 125I-labeled anti-mouse whole antibody from sheep (100,000 cpm/ml; Amersham Corp., Arlington Heights, IL) was used as a second antibody to develop the Western blots. After washing, the blots were exposed overnight to X-OMAT X-ray film (Kodak, Rochester, NY), at −70°C, using intensifying screens. The level of phosphorylated EGF receptor was determined by densitometric scanning of the autoradiogram.
INHIBITION OF BREAST CANCER CELL GROWTH BY RG-13022

Fig. 3. Effect of RG-13022 concentration on autophosphorylation of the EGF receptor in MDA-231 cells. Cells were grown as shown in Fig. 2, incubated for 16 h with increasing concentrations of RG-13022, stimulated for 5 min with EGF (10 nM), and lysed. EGF receptor phosphorylation was evaluated by Western blot analysis. The figure shows the results of densitometric scanning of the autoradiogram (inset), expressed as percentage of control cells.

Fig. 4. Effect of RG-13022 on EGF-stimulated cell proliferation. MCF-7 cells were plated in IMEM with 10% serum. Twenty-four h later the seeding medium was removed, the cells were washed twice with PBS, and the medium was then changed to phenol red-free and serum-free IMEM, with or without inhibitor. The medium was replaced with fresh medium on day 3. The cells were counted on day 5. Bars, mean ± SE of triplicate determinations.

addition to its effect on EGF-mediated growth. Similar data were observed with T47D cells (data not shown).

Cells growing under these conditions in the presence of RG-13022 (5 μM) appeared to remain viable, as indicated by their morphological appearance, by the lack of floating cells, and by the ability of the cells to exclude trypan blue (>90% viable). These data suggested that the inhibitory effect of the tyrosine kinase inhibitor was cytostatic rather than cytotoxic at this concentration. To further investigate whether the effect of RG-13022 was lethal, we determined the reversibility of its inhibitory effect. A prolonged time course of the effect of RG-13022 on [3H]thymidine incorporation in MCF-7 cells is shown in Fig. 5. Inhibition was observed within 1 h of the addition of the inhibitor, and the effect was maximal by 3 h, at a concentration of 1 μM (Fig. 5A). At this concentration the inhibitory effect was temporary, and after 20 h DNA synthesis began to increase. At a concentration of 5 μM (Fig. 5B) the inhibitory effect was more dramatic, and DNA synthesis remained blocked for at least 48 h. Similar data were obtained with T47D cells (data not shown). Fig. 6 demonstrates that the inhibitory effect is reversible by replacement of RG-13022-containing medium with fresh medium. T47D cells were grown in the presence of 1 or 5 μM RG-13022 for 5 days. At that time, some culture dishes received fresh medium, while others received fresh medium supplemented with the same concentration of the tyrosine kinase inhibitor. The inhibitory effect of 1 μM RG-13022 was reversible, and cells proliferated at a faster rate when the inhibitor was removed from the medium. With 5 μM RG-13022 the inhibitory effect was minimally reversible, and it persisted for at least several days after removal of the inhibitor from the medium.

We next examined the effects of the tyrosine kinase inhibitor on a panel of ER+ and ER- breast cancer cell lines growing in medium supplemented with serum (Fig. 7). RG-13022 at a concentration of 5 μM inhibited [3H]thymidine incorporation in the three ER+ cell lines (T47D, MCF-7, and ZR-75-1), as well as in the two ER- cell lines (BT-20 and MDA-231).

Inhibition of serum-stimulated cell proliferation suggested that the effects of RG-13022 might not be EGF specific. To test this possibility, we examined the ability of RG-13022 to inhibit the stimulatory effects of several different polypeptide growth factors (Fig. 8). RG-13022 inhibited EGF-stimulated growth in a dose-dependent fashion. The concentration of RG-13022 required to inhibit DNA synthesis by 50% of maximum
INHIBITION OF BREAST CANCER CELL GROWTH BY RG-13022

Fig. 7. Effects of RG-13022 on a panel of breast cancer cell lines. ER* cell lines (MCF-7, T47D, and ZR-75-1) and ER- cell lines (MDA-231 and BT-20) were grown in IMEM with serum, in the presence or absence of 5 μM inhibitor. [%H]Thymidine incorporation was measured after 16 h. The data are expressed as percentage of control values. Bars, mean of triplicate determinations. The SE were all <5%. All differences are statistically significant.

Table 1 Effect of RG-13022 on the growth of MCF-7 and CHO cells

Cells were grown in the regular medium, with serum, for 24 h. The seeding medium was removed and cells were washed twice with PBS and then changed to regular medium with 1% charcoal-stripped serum, in the presence of EGF (10 nM) alone or with the inhibitor (5 μM). The medium was replaced with fresh medium on day 3. The cells were counted on day 5. The values represent the mean ± SE of triplicate determinations.

<table>
<thead>
<tr>
<th>Cell number (× 10^6)</th>
<th>EGF alone</th>
<th>EGF + RG-13022</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>1.29 ± 0.07</td>
<td>0.8 ± 0.07*</td>
</tr>
<tr>
<td>CHO cells</td>
<td>1.93 ± 0.15</td>
<td>2.0 ± 0.14*</td>
</tr>
</tbody>
</table>

* P = 0.007.
* P = 0.075.

(2 μM) was similar to that causing half-maximal inhibition of EGF receptor autophosphorylation. However, the inhibitory effects of RG-13022 were not specific for EGF. Not only were the stimulatory effects of EGF and TGF-α inhibited in a dose-dependent manner, but stimulation by IGF-I, IGF-II, or insulin was also significantly inhibited. These data suggest either that this tyrosine kinase inhibitor can inhibit the tyrosine kinase of several different growth factor receptors or that it inhibits a more distal event common to all of these mitogenic pathways. On the other hand, RG-13022 was not nonspecifically cytotoxic for all cells (Table 1). Proliferation of CHO cells was not affected by RG-13022 at a concentration that significantly inhibited growth of MCF-7 cells.

Effect of RG-13022 on Estrogen-induced Growth. Estrogen treatment of ER* human breast cancer cells has been reported to increase the synthesis and secretion of several polypeptide growth factors, including IGF-II and TGF-α, which may then regulate growth in an autocrine fashion (1, 11, 18–20, 21). If estrogen-stimulated TGF-α expression is sufficient to induce a biological response, then estrogen treatment should increase autophosphorylation of EGF receptors via increased secretion of this growth factor. Fig. 9 demonstrates that both EGF and estrogen markedly increased phosphorylation of the M, 170,000 EGF receptor in T47D cells, an effect that was blocked by RG-
13022. (A similar study could not be performed in MCF-7 cells because of the lower EGF receptor content of this line.) To determine whether RG-13022 can inhibit estrogen-induced growth, \[^{3}H\]thymidine incorporation and cell number were analyzed in both T47D and MCF-7 cells growing in the presence of EGF or estrogen, with or without RG-13022. Fig. 10 shows illustrative experiments in T47D cells (Fig. 10A) and MCF-7 cells (Fig. 10B). As shown earlier, EGF-stimulated thymidine incorporation and cell proliferation were significantly blocked in both cell lines by the addition of RG-13022. Interestingly, estrogen-stimulated growth of both cell lines was also totally blocked by the tyrosine kinase inhibitor. In a separate dose-response experiment, RG-13022 inhibited estrogen-induced growth at concentrations paralleling those producing its effects on EGF-stimulated growth (data not shown).

**DISCUSSION**

Cancer and other hyperproliferative diseases are associated with elevated protein kinase activity, partially related to the increased activity of growth factor receptors. It has been hypothesized that chemical blockers, particularly of the substrate domain of the receptor protein tyrosine kinase, might prove to be effective and potentially selective inhibitors of cellular proliferation in diseases, like breast cancer, that are regulated by a host of hormones and growth factors (12, 17, 18).

The first class of tyrosine kinase inhibitors described, called tyrphostins, had some selectivity for the EGF receptor (6); they were several hundred-fold less effective in inhibiting the kinase activity of the insulin receptor. Furthermore, these tyrphostins were more potent inhibitors of EGF-stimulated cell proliferation than of serum-stimulated cell proliferation. The tyrphostins had no effect on receptor number, EGF binding, or EGF-induced receptor internalization and down-regulation (7).

In the present study, we have found that the new tyrosine kinase inhibitor RG-13022, which is more selective for the EGF receptor than are the prototype inhibitors, inhibited the EGF receptor tyrosine kinase activity in cultured human breast cancer cells. RG-13022 was also a potent inhibitor of EGF-induced cell proliferation. Similar to other reports using other analogues, the effect of RG-13022 was largely cytostatic and reversible when the drug was removed from the culture medium (6, 7). We cannot exclude the possibility that “reversibility” following removal of RG-13022 from the medium could be related to selection of resistant clones during the treatment period.

The growth-inhibitory effects of RG-13022 were not specific for the EGF receptor pathway. This tyrosine kinase inhibitor was a potent inhibitor of serum-stimulated growth of several ER\(^+\) and ER\(^-\) breast cancer cell lines, an effect that could be partially explained by blockade of the autocrine effects of TGF-\(\alpha\) secreted by these cell lines or of EGF present in serum (11). However, RG-13022 also inhibited growth stimulated by several unrelated growth factors, including insulin, IGF-I, and IGF-II, at doses similar to those required to inhibit EGF-induced growth. It is possible that, in intact cells, the tyrosine kinase activity of other growth factor receptors or other tyrosine kinases critical to rapidly proliferating cells are also inhibited by this agent. RG-13022 is known to inhibit the tyrosine kinase activity of the platelet-derived growth factor and insulin receptors, albeit at higher concentrations. Its effects on other growth factor receptors or signal transduction pathways remain to be defined. Further studies on the effects of RG-13022 on other pathways are necessary to answer this question.

Interestingly, RG-13022 also inhibited estrogen-induced growth of MCF-7 and T47D breast cancer cells. It is possible that this tyrosine kinase inhibitor may nonselectively inhibit cell proliferation, although CHO cells that lack EGF receptors were unaffected by similar concentrations of the drug. It is more likely that functioning TK pathways are required for estrogen-induced growth. Several groups have shown that estrogen treatment of these cells increases the expression and secretion of growth factors such as TGF-\(\alpha\) and IGF-II (1, 20, 21), which could be partial mediators of estrogen-induced growth. We have questioned this hypothesis by showing that antibody blockade of the surface membrane EGF receptor and/or the IGF-I receptor fails to inhibit estrogen-induced growth (11, 18), apparently contradicting our results with RG-13022. However, an alternative explanation is that these autocrine growth factors can interact with their receptors inside the cell and stimulate proliferation via internal autocrine loops that have been observed for other growth factors (22, 23). These internal loops would not be accessible to inhibition by receptor-blocking antibodies but would be inhibited by RG-13022, which freely enters the cell. It is also possible that RG-13022 inhibits the tyrosine phosphorylation of the estrogen receptor, which may be critical for its ability to bind hormone (24). These alternative possibilities require additional study.

Our data do show that tyrosine kinase inhibitors can inhibit proliferation of breast cancer cells. Several tyrosine kinase inhibitors have also recently been shown to inhibit in vitro growth and in vivo growth, in nude mice, of a human squamous cell carcinoma (25). Importantly, administration of RG-13022 to mice twice daily for 6 weeks, at doses that inhibited tumor growth, had no apparent toxicity. These data suggest that the increased tyrosine kinase activity associated with cancer cells is a worthwhile target for additional study and that tyrosine kinase inhibitors may provide a new strategy for the endocrine therapy of cancer.

**REFERENCES**

11. Arteaga, C. L., Coronado, E., and Osborne, C. K. Blockade of the epidermal growth factor receptor inhibits transforming growth factor alpha-induced but...
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