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Antitumor Effect of the Combination of CGS16949A and 5-FU against Human Breast Cancer Cell Lines

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Abstract : The aim of this study was to evaluate the combined antitumor effect of CGS16949A and 5-fluorouracil (5-FU) against human breast cancer cell lines. MCF-7, SK-BR-3, and MDA-MB-231 were cultured and measured with [³H] water assay and MTT assay. Three human breast cancer cell lines were treated with 10 nM CGS16949A and 100 μ g/ml 5-FU. The aromatase activities (percent aromatase activity) of the three human breast cancer cell lines were significantly inhibited by CGS16949A. In particular, combined treatment with CGS16949A and 5-FU inhibited aromatase activities more than did CGS16949A alone. In two cell lines (SK-BR-3 and MDA-MB-231) inhibition rates increased with 5-FU in a dose-dependent manner, and, in particular, the combined antitumor effect of CGS16949A and 5-FU against SK-BR-3 with the MTT assay was shown. To our knowledge, this is the first report of a combined antitumor effect of an aromatase inhibitor (CGS16949A) and 5-FU in three human breast cancer cell lines.

Key words : breast cancer, cell lines, aromatase, CGS16949A, fluorouracil

Introduction

Aromatization of breast carcinoma has been found to occur to a greater degree in postmenopausal women, in whom the most important source of estrogens, the ovary, is absent^{1,2)}. Aromatase is present in human breast tumors and in breast cancer cell lines, a finding that suggests the possibility of in-situ estrogen production via androstenedione to estrogen³⁾. CGS16949A (Fadrozole, Novartis, Basel, Seitzerland) is a nonsteroidal competitive inhibitor of aromatase and plays an important role in the blocking of estrogen synthesis⁴⁾. In postmenopausal women, aromatase inhibitors are reported to be useful for the treatment of breast cancer^{5,6)}. 5-fluorouracil has been widely used in the adjuvant chemotherapy for women with advanced breast cancer with or without estrogen receptors. The antitumor effect of CGS16949A or 5-FU has been investigated⁷⁻¹⁰⁾; however, the effect of the combination CGS16949A and 5-FU has not been reported. The purpose of this study was to investigate the antitumor effect of the combination of CGS16949A and 5-FU against three human breast cancer cell lines in vitro.

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Materials and Methods

Cell lines

The human breast cancer cell lines MCF-7, SK-BR-3, and MDA-MB-231 were purchased from the American Type Culture Collection (Rockville, MD, USA). The three cell lines were grown in 25-cm² tissue culture flasks, but the culture media and cell culture conditions were different. MCF-7 was grown in Eagle's minimum essential medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) with nonessential, nonacidic, phenol red, and 10 % fetal calf serum (FCS) plus 1 mM sodium pyruvate (Gibco BRL, Grand Island, NY, USA) and 10 μ g/ml bovine insulin (Gibco BRL). SK-BR-3 was grown in McCoy's 5a medium (Gibco BRL) containing 10 % FCS. MDA-MB-231 was grown in Leibovitz's L-15 medium (Gibco BRL) with 10 % FCS. MCF-7 and SK-BR-3 were incubated at 37 °C in air with 5 % CO₂. MDA-MB-231 was incubated at 37 °C in a closed system without CO₂. The media of all cell cultures were changed every 3rd day. Estrogen receptors have been reported in MCF-7 and SK-BR-3 but not in MDA-MB-231.

Chemicals

CGS16949A was provided by Novartis. 5-FU was purchased from the Kyowa Hakko Co., Ltd (Tokyo, Japan) in an injectable form. [1β -³H] Androstenedione (24.70 Ci/mmol) was purchased from New England Nuclear Corp. (Boston, MA, USA). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Aromatase activity assay

Aromatase activity in the three cell lines was measured with the tritiated water method as previously described^{11,12}. Briefly, cells were seeded in duplicate at a density of 1×10^5 cells/dish in 5 ml feeding medium in 60-mm Petri dishes. The cells were grown for 1 week until confluent in medium. Preconfluent cells were incubated with [1β -³H] androstenedione as a substrate to a concentration of 100 nM at 37 °C in air with 5 % CO₂ for 5 hours. After incubation for 5 hours, 1.5 ml of culture medium was transferred to a test tube, to which 0.2 ml of 30 % trichloroacetic acid and 0.5 ml of 10 % charcoal were added and mixed. The mixture was incubated at 37 °C in air for 30 minutes. The mixture was centrifuged, and the supernatant was filtered through a cotton-plugged Pasteur pipette. The amount of [³H] water in the eluate was assessed on the basis of the theory that [1β -³H] is eliminated during aromatization of the [1β -³H] androstenedione substrate (75 % release into water). The [³H] radioactivity in medium was measured with a liquid scintillation counter (Aloka LSC-651, Tokyo, Japan). Ten nanomoles of CGS16949A alone, 100 μ g/ml of 5-FU alone, or a combination of CGS16949A and 5-FU were added to investigate their inhibitory effects on enzymatic activities. Protein content was measured with the protein-dye binding method (Tonein-TP, Ozuka, Japan)¹³.

Chemosensitivity test (MTT assay)

MTT was measured with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, succinic acid, and dimethyl sulfoxide (DMSO). Subconfluent cultures of breast cancer cell lines were trypsinised and washed twice with the medium. Single cell suspensions with 20 % FCS were seeded at a density of 1×10^4 cells/well in 50 μ l nutrient medium with or without phenol red in 96-well plates. In cultures treated with CGS16949A alone, predetermined concentrations (10 and 100 nM) of CGS16949A dissolved in ethanol were dried, then diluted with the medium with or without phenol red. In cultures treated with

CGS16949A and 5-FU, CGS16949A (10 nM) dissolved in ethanol was dried, then diluted with 5-FU, including predetermined medium concentrations for 10, 100, and 1000 μ g/ml. In cultures treated with 5-FU alone, predetermined concentrations (10, 100, and 1000 μ g/ml) of 5-FU were diluted directly with the culture medium. Then the medium was added at 50 μ g/well in quadruplicate to achieve a total volume of 100 μ g/well. After incubation for 72 hours as above, 10 μ l of MTT and succinic acid were added to each well. The plates were incubated for 3 hours, and 200 μ l 100 % dimethyl sulfoxide was added to each well.

The plates were read at an absorbance of 560 nm on a MTP-120 microplate reader (Corona Electric Co., Ltd., Ibaragi, Japan). The inhibition rate was calculated according to the equation: inhibition rate = $[(a-p)/(a-m)] \times 100(\%)$

a: control absorbance units

m: background absorbance units

p: treatment absorbance units

Statistical analysis

Fisher's protected least significant difference (PLSD) test was used to examine the statistical significance of change in inhibition rates, which are expressed as the mean $\pm$ standard deviation (SD).

Results

Aromatase activity assay

The aromatase activities of MCF-7, SK-BR-3, and MDA-MB-231 without putative inhibitors were 106, 3250, and 132 fmol/hour/mg protein, respectively. The aromatase activity of MCF-7 was inhibited 26 %, 63 %, and 22 % in the presence of 10 nM CGS16949A, 100 μ g/ml 5-FU, and a combination of CGS16949A and 5-FU, respectively. The aromatase activity of SK-BR-3 cells was inhibited 15 %, 73 %, and 11 % in the presence of 10 nM CGS16949A, 100 μ g/ml 5-FU, and a combination of CGS16949A and 5-FU, respectively. The aromatase activity of MDA-MB-231 cells was inhibited 58 %, 78 %, and 63 % in the presence of 10 nM CGS16949A, 100 μ g/ml 5-FU, and a combination of CGS16949A and 5-FU, respectively (Fig. 1).

MTT assay

For the MCF-7 cell line, the inhibition rate with 10 and 100 nM CGS16949A was estimated at 10.2 % and 12.8 %. The inhibition rate was less than 50 % even with 1000 μ g/ml 5-FU alone. The inhibition rate was estimated to be greater than 50 % only when both 1000 μ g/ml 5-FU and 10 nM CGS16949A were administered. For the SK-BR-3 cell line, the inhibition rate with 10 and 100 nM CGS16949A was estimated at 35.8 % and 29.5 %. The sensitivity of SK-BR-3 to CGS16949A was higher than that of MCF-7. The inhibition rate with 5-FU alone increased in a dose-dependent manner and reached 48 % with 100 μ g/ml 5-FU. The inhibition rate with 10 nM CGS16949A and 100 μ g/ml 5-FU was 60 %, similar to that with 1000 μ g/ml 5-FU alone. For the MDA-MB-231 cell line, the inhibition rate with 10 or 100 nM CGS16949A was approximately 15 % and similar to that for MCF-7 cells. However, for the MDA-MB-231 cell line, the inhibition rate with 5-FU increased in a dose-dependent manner and was 29 % with 100 μ g/ml 5-FU. In contrast, the combination of 10 nM CGS16949A and 100 μ g/ml 5-FU did not effectively inhibit the MDA-MB-231 cell line (Fig. 2).

The inhibition rates of the three human breast cancer cell lines are shown in Fig. 3.

Fig. 1. The effect of aromatase inhibitor CGS16949A and 5-FU on aromatase activity in human breast cancer cell lines as measured with the [^3H] water release method.
 □, Control; ■, CGS16949A (10 nM); ▨, 5-FU (100 $\mu\text{g/ml}$); and ▩, CGS16949A (10 nM) and 5-FU (100 $\mu\text{g/ml}$).

For the MCF-7 and SK-BR-3 cell lines, the inhibition rates were significantly higher with 10 nM CGS16949A and 100 $\mu\text{g/ml}$ 5-FU than with 10 nM CGS16949A alone. The combination of CGS16949A and 5-FU produced inhibition rates of more than 50 % and was the only treatment to have an antitumor effect against the SK-BR-3 cell line.

Discussion

While the antitumor effect of the aromatase inhibitor CGS16949A on human breast cancer cell lines and the antitumor effect of 5-FU against breast cancer cell lines have been reported¹⁴⁻¹⁶⁾, the effect of the combination of CGS16949A and 5-FU on human breast cancer cells has not yet been reported. In this study, we investigated the antitumor effect of CGS16949A or 5-FU alone and the combined antitumor effect of CGS16949A and 5-FU against human breast cancer cell lines with [^3H] water assay and MTT assay.

CGS16949A is a nonsteroidal competitive inhibitor of aromatase⁴⁾. Yano et al.¹⁴⁾ reported that the aromatase activity of MCF-7 is inhibited by CGS16949A in a dose-dependent manner, which suggests that one of the growth-inhibiting mechanisms of CGS16949A is the inhibition of intracellular aromatase activity. In our study, the aromatase activity of three human breast cancer cell lines was significantly inhibited by 10 nM CGS16949A, and inhibited to a lesser extent by 100 $\mu\text{g/ml}$ 5-FU. The aromatase activity of these cells was inhibited more effectively by a combination of 10 nM CGS16949A and 100 $\mu\text{g/ml}$ 5-FU than by each agent alone. Dowsett et al.¹⁵⁾ reported that cytotoxic chemotherapy suppresses aromatase activity in breast carcinomas in vivo. Cytotoxic chemotherapy might preferentially lead to the death of aromatase-positive cells. MTT assay in the present study showed that the inhibition rates of 5-FU increased in a dose-dependent manner in SK-BR-3 and MDA-MB-231. The combination of 10 nM CGS16949A and 100 $\mu\text{g/ml}$ 5-FU had an antitumor effect against SK-BR-3 which was as great as that with 1000 $\mu\text{g/ml}$ 5-FU alone.

Fig. 2. The effect of aromatase inhibitor CGS16949A and 5-FU, given alone or in combination, on cell proliferation in MCF-7, SK-BR-3, and MDA-MB-231 cell lines as measured with MTT assay. Bars represent mean $\pm$ SD. *Indicates $P < 0.05$, by Fisher's PLSD.

These results suggest addition of CGS16949A and 5-FU might allow doses of 5-FU to be reduced.

In our pilot study, flow cytometry showed that the percentage of cells in the S-phase in each cell line was different. The percentages of cells in S-phase in MCF-7, SK-BR-3, and MDA-MB-231 were 14.02 %, 21.21 %, and 29.05 %, respectively. SK-BR-3 and MDA-MB-231 showed a higher percentage of S-phase cells than did MCF-7. 5-FU has been shown to act more effectively on cells in S-phase¹⁷⁾. The results of the MTT assay suggest a specific relationship between 5-FU and the S-phase. Because CGS16949A is an imidazole derivative of aminoglutethimide, aminoglutethimide affects the G0/G1 fraction of cell nuclei in the MXT mouse mammary tumor in vivo¹⁸⁾. In this study, combined treatment with CGS16949A and 5-FU was most effective against SK-BR-3 cells, a finding that suggests that combination therapy might be effective as a postoperative adjuvant therapy against breast cancers that have high aromatase activity and a high percentage of cells in S-phase.

Fig. 3. The effect on cell proliferation of CGS16949A (10 nM) and 5-FU (100 μ g/ml), given alone or in combination in MCF-7, SK-BR-3, and MDA-MB231 cell lines, as measured with MTT assay. Bars represent mean $\pm$ SD. *Indicates $P < 0.05$, by Fisher's PLSD.
 □, Control; ■, CGS16949A (10 nM); ▨, 5-FU (100 μ g/ml); ▩, CGS16949A (10 nM) and 5-FU (100 μ g/ml).

Recently, hormonal agents have become the treatment of choice for most postmenopausal patients with advanced breast cancer, and aromatase inhibitors have achieved satisfactory therapeutic results when used as a first-line treatment¹⁹⁾. The combination of hormonal agents and 5-FU was often used without reference to hormone receptor status as an adjuvant therapy after surgery. In this study, combined treatment with CGS16949A and 5-FU was most effective against the SK-BR-3 cell line, a finding that suggests that combination therapy might be effective as a postoperative adjuvant therapy against breast cancers that have high aromatase activity. Furthermore, the combination may also allow the effective dose and side effects of 5-FU to be reduced.

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