Abstract. Aim: To determine a novel chemotherapeutic concept for hormone receptor-negative and HER2-positive breast cancer, a high progression form of the disease for which treatment has been difficult. A combination therapy of 5-fluorouracil (5-FU) with rapamycin (Rap) was examined.

Materials and Methods: The growth inhibitory effect of treatment was evaluated by an MTT assay and cellular signal/apoptotic pathways were investigated by Western blotting and Hoechst 33342 staining. Results: Rap was shown to induce an inhibitory effect on the phosphorylation of mTOR and p70S6K. The expression of thymidine synthase (TS) was decreased by Rap. The addition of 5-FU to Rap was found to increase cell death. The Hoechst 33342 assay showed that apoptosis was increased by the combination of 5-FU and Rap in comparison to 5FU alone. Conclusion: 5-FU is more effective in combination with the TS-reducing action of Rap, even for highly HER2-expressing breast cancer cells.

Among women, breast cancer is the most common malignancy in the United States and Japan, and still a highly frequent cause of cancer-related death (1). In 2008, 11,797 breast cancer deaths among women occurred in Japan (2). For this problematic cancer, knowledge of the expression of hormone receptors, the oestrogen receptor (ER) and progesterone receptor (PgR), are critical in order to decide a treatment strategy. Oestrogens are not only the main regulators of growth and differentiation in the normal mammary gland, but also play a major role in the onset and progression of breast cancer (3), and their presence is a good predictor of response to endocrine agents (4, 5). Most breast cancers, particularly those of postmenopausal women, are hormone receptor-positive, and according to the Early Breast Cancer Trialists’ Collaborative Group (EBCTCG), the standard recommendation has been therapy with a selective oestrogen receptor modulator (6). However, clinically, hormone receptor (ER)-negative breast cancer has been detected in 37.8% of patients (7); therefore, for these patients, a novel treatment strategy should be investigated. Molecular targeting therapy has emerged in various fields of cancer treatment (8), especially for breast cancer, as epidermal growth factor (EGF) related-receptor family (HER2) targeting drugs, monoclonal antibodies and receptor inhibitors have been employed (9, 10).

Indeed, routine measuring for the presence of HER2 in combination with ER and PgR is now recommended (11, 12). HER2 is overexpressed in approximately 20% of breast cancer patients and is considered the main driving force for tumour progression (13). Therefore, HER2-positive breast cancer represents a particularly aggressive tumour subset, with increased proliferation and metastatic potential (14, 15), and induce poor prognosis (16). In recent years, HER2-focused chemotherapy has been applied to patients with HER2-positive tumours, but cases resistant to the treatment have been detected (17). In the present study, to improve treatment of patients with hormone-negative and HER2-positive breast cancer, a key drug that is a standard option for treating solid malignancies was applied, namely 5-fluorouracil (5-FU) (18), and enhancement of its action via an effector was studied in cell lines of breast cancer cases with poor therapy options and aggressive tumour invasion.

Materials and Methods

Cell culture. The human breast cancer cell line, SKBR3 (ATCC, Manassas, VA, USA), which is ER/PgR negative and HER2 positive, was selected. Cells were maintained in DMEM (Sigma, St. Louis, MO, USA), supplemented with 10% foetal bovine serum (Sigma), 2% L-glutamine (MP Biomedicals, Eschwege, Hesse, Germany), 2% sodium pyruvate (Sigma), 1% MEM non-essential amino acid solution (Sigma), 1% antibiotic/antimyotic solution (Sigma), and 0.1% tylosin solution (Sigma) in 5% CO2 and 95% air at 37°C.

MTT assay. Cellular proliferation of the breast cancer cell line was evaluated by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) method, as described previously (16, 17). MTT (Sigma) (5 mg/ml) was dissolved in PBS and the solution was stored at 2-8°C for frequent use, after filtration through a 0.2 μm filter. Briefly, cells (1×10⁶) were cultured in 96-well plates for
The medium was then removed, and the cells were treated with several concentrations (0.1-10 mM) of 5-FU (Wako, Osaka, Japan) or 10 μM rapamycin (Rap) (Wako) for 24 h. After being washed with PBS, the cells were incubated in MTT solution for 0.5 h. Following dissolution of MTT with dimethylsulfoxide (DMSO: Wako), the absorbance of the resulting solution was measured using a Microplate reader (Bio-Rad, Hercules, CA, USA) at a wavelength of 540 nm. The absorbance of the solution from the control cells was designated as 100%. The experiments were performed in triplicate, and the mean and standard deviation (SD) of the data were calculated. Statistical analysis was performed using Student’s t-test. A p-value <0.05 was considered statistically significant.

**A apoptotic assay with Hoechst 33342.** Cells (2.7×10⁵) collected with a cell scraper were cultured on chamber slides for 24 h. After the medium was removed, the cells were treated with several concentrations of 5-FU and Rap for 24 h. After being washed with PBS, the cells were fixed by an acetic acid/methanol solution (mixing proportion: 1:3 by volume) for 20 min and stained with Hoechst 33342 stain solution (Dojindo Laboratories, Kumamoto, Japan) at room temperature for 10 min. With a drop of mounting solution (VectaShield, Vector laboratories, Inc., Burlingame, CA, USA), the cells were analysed by fluorescence phase-contrast microscopy. The apoptotic index (AI) was calculated following evaluation of 1,000 cells in each sample, as described previously (19).

**Western blotting.** Cells (3.8×10⁶) treated with various concentrations of 5-FU (0.1-10 mM) and 10 μM Rap were lysed in RIPA buffer (150 mM NaCl, 1.0% NP40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris (pH 8.0)) containing phosphatase inhibitors (Sigma) and protease inhibitors (Sigma). Twenty μg of protein from each sample were electrophoresed through a polyacrylamide-SDS gel (Ready Gels J, Bio-Rad) in buffer (10×Tris/glycine/SDS buffer; Bio-Rad), and blotted on PVDF membranes (Immobilon-P Transfermembrane; Millipore, Billerica, MA, USA) in transfer buffer (10×Tris/glycine buffer; Bio-Rad). The proteins were exposed to a monoclonal antibody against caspase-3 (Cell Signaling, Boston, MA, USA), thymidylate synthase (TS) (Chemicon, Billerica, MA, USA), orotate phosphoribosyltransferase (OPRT) (Taiho Pharmaceutical, Tokyo, Japan), cyclinB1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), cyclinD1 (Calbiochem, Darmstadt, Hessen, Germany), beta actin-loading control (Abcam, Tokyo, Japan), mammalian target of Rap (mTOR) (Cell Signaling), phospho-mTOR (ser2481) (Cell Signaling), phospho-p44/42 mitogen-activated protein kinase (MAPK) (Cell Signaling) and phospho-AKT (Cell Signaling), and the interaction was detected with the enhanced chemiluminescence (ECL) system (Western Lighting Plus-ECL, PerkinElmer Inc., Waltham, MA, USA) after the addition of anti-mouse or anti-rabbit IgG (GE Healthcare, Buckinghamshire, England), as described previously (20-22).

**Results**

Rap-induced cellular responses. The Rap-induced cellular signalling pathway was evaluated by Western blotting (Figure 1). Rap was shown to induce an inhibitory effect on phosphorylation of mTOR (ser2481) and p70S6K for 2 hours at concentrations over 5.0 μM. The inhibition of the phosphorylation of mTOR and p70S6K was dependent on the dose and time. The accumulation of cyclin B1 for 6 hours was noted at concentrations of Rap over 1.0 μM, despite the lack of any difference in the detection of cyclin D1 until 72 hours (data not shown). In addition, the expression of thymidine synthase (TS) but not OPRT or dihydropterymidine dehydrogenase (DPD), was reduced for 48 hours by the addition of 5.0 μM Rap.

Rap induced another signaling action that was examined; it was found to phosphorylate extracellular signal-regulated kinase (ERK) and AKT at concentrations over 0.1 μM (Figure 2), but had no effect on other MAPK factors (data not shown). Additionally these phosphorylations were inhibited by the non-thiol antioxidant, catalase, but not the thiol antioxidant, L-cysteine. In contrast, the decrease in mTOR or TS expression was not associated with the presence of antioxidant (data not shown). U0126 as a potent ERK inhibitor antagonised Rap-induced phosphorylation of ERK, but not AKT.

**Combinatory actions of 5-FU and Rap.** SKBR3 breast cancer cells were exposed to several concentrations (0. 1-10 mM) of 5-FU with or without 10 μM Rap for 72 h (Figure 3). 5-FU induced cell toxicity in a dose dependent manner, and IC₅₀, the 5-FU concentration corresponding to 50% cell viability, was calculated as 5.1±0.2 mM. Rap at 10 μM had almost no growth inhibitory effect, while the addition of 5-FU was found to mediate further cell death, with a significantly lowered IC₅₀ (p=0.004) calculated as 0.25±0.03 mM.

The apoptotic pathway related to caspase, which belongs to the cysteine protease group and is involved in signal transmission during programmed cell death, was studied (23) (Figure 4). Rap itself had no effect on activating caspase-3, while 5-FU induced caspase-3 cleavage, which is the activated form. The concentration of 5-FU that mediated caspase-3 activation was in excess of 1.0 μM when used individually, and was only 0.1 μM in combination with Rap. As shown in Figure 5 in the Hoechst 33342 assay, AI was shown to be increased by 5-FU, but not by Rap, while it was more clearly noted that it was high when the agents were combined (AI: 0.018 vs. 0.053, for 5-FU only and the combination of 5-FU with Rap, respectively).

**Discussion**

5-FU and its derivatives have been widely used and are considered to be key drugs in the treatment of solid malignancies, including breast cancer. However, because single-agent response rates are only 10-30% (24), finding new combinations with anticancer drugs for high therapeutic effects has provided an important new approach to treatment (25). Under this concept, Rap, which is a macrolide derived from Streptomyces hygroscopicus and has been in clinical use as immunosuppressant, is currently being assessed for the treatment of advanced cancer (26). To evaluate the
combination therapy with 5-FU and Rap for breast cancer, the mechanism was observed in the present study using biological approaches.

Cyclins are a group of proteins involved in cell cycle regulation. Cyclin D1 is synthesised in the pre-DNA-synthetic gap (early G1 phase) just before the induction of S phase, while cyclin B1 is produced from the late G2/M phase and is degraded during M phase (27). Most Rap-treated cells are arrested specifically in the G1 phase of the cell cycle, and Rap-induced arrest of the cell cycle is reversed by cyclin D1 over-expression, suggesting that cyclin D1 is a key mediator of proliferation downstream of mTOR (28). However, cyclin D1 is not down-regulated in all cells, which show a growth inhibitory response, and other studies have found that Rap specifically disrupts the formation of cyclin D1-related proliferating cell nuclear antigen complexes, thereby inhibiting cell cycle progression (29). In the present study, changes in cyclin D1 following treatment of cells with Rap were not noted and the increased expression of cyclin B1 was associated with cell-cycle blockage at G1, which appears to be critical to the effect of Rap treatment in breast cancer cells. At the same time, reactive oxygen species (ROS) are generated in aerobic organisms during biochemical process, as well as in response to various endogenous or exogenous stimuli. The toxic effects of oxidative stress depend upon the degree of stress and/or the type of cell (30). Among the ROS-induced signal pathway-related proteins, the MAPK family represents an important factor for cellular response. C-Jun-N-terminal kinases and p38 MAPK are activated by environmental stress and are closely associated with cell death, whereas ERK is activated by growth factors and has an effect in preventing cell deaths (31). Therefore, Rap-induced ERK phosphorylation was completely inhibited by antioxidant suggesting the possibility of interaction with ROS. The ERK inhibitor, U0126, has no effect on activating AKT, despite blocking ERK phosphorylation completely, which indicates that the

![Figure 1. Rapamycin induced cellular signaling. a: The expression of the phosphorylated type of mTOR and s70S6K was reduced by 10 μM Rapamycin (Rap) in a time-dependent manner. b: The expression of the phosphorylated type of mTOR and s70S6K was reduced by Rap in a dose-dependent manner. A dose-dependent decrease in TS was found after the induction of Rap. However, it was not found in OPRT. Accumulation of cyclin B1 was noted at concentrations of Rap over 0.1 μM.](image1)

![Figure 2. The mechanism of Rapamycin-induced AKT-related signal activation. a: Rapamycin (Rap) at concentrations over 0.1 μM induced the expression of phospho-ERK and phosphor-AKT after 0.5 hour. b: These phosphorylations were inhibited by the non-thiol antioxidant, catalase, but not by the thiol antioxidant, L-cysteine. c: U0126, as a potent ERK inhibitor, antagonised Rap-induced phosphorylation of ERK, but not AKT.](image2)
ERK-AKT pathway is independent of Rap-mediated actions. A recent report showed that mTOR was reduced in conditions of hypoxia (32), but in the present study, oxidative stress had no correlation with mTOR induction.

The phosphatidylinositol-3-kinase (PI3K) pathway has been reported as one of the important intracellular mediators frequently activated in cancer cells, including HER2-related signals (33). PI3K activates a number of signaling molecules, among which the AKT/mTOR pathway is of particular interest because of its role in inhibiting apoptosis and promoting cell proliferation (34). Activation of mTOR proceeds via two distinct signaling complexes: TORC1 and TORC2 (35). TORC1 is Rap-sensitive and is implicated in proteins such as p70S6K of growth factor signalling, and TORC2 has been demonstrated as a Rap-sensitive complex, which phosphorylates AKT (36). AKT is both upstream and downstream of mTOR, but the present study demonstrated that AKT activation may be independent from the mTOR or MAPK pathway, because it was inhibited by antioxidant but not by U0126. On the other hand, mTOR has also been shown to regulate protein synthesis and cell cycle progression (37). Cell culture studies demonstrated that the mechanisms of mTOR controlling protein synthesis were associated with phosphorylating downstream substrates, such as p70S6K (38). The targeting proteins were indicated to include TS, but not DPD and OPRT, all of which are key enzymes in the regulation of 5-FU metabolism to determine the effect of the agent on cancer cell (39). 5-FU itself needs to be metabolised by OPRT to become active, and DPD returns it to an inactive state. In addition, the TS complex with other coenzymes blocks the functional effect of 5-FU to inhibit DNA synthesis.

5-FU itself needs to be metabolised by OPRT to become active, and DPD returns it to an inactive state. In addition, the TS complex with other coenzymes blocks the functional effect of 5-FU to inhibit DNA synthesis.
Therefore, under conditions of high OPRT and low DPD/TS activities, chemotherapy with 5-FU facilitates a more effective response. The present study indicated that Rap itself had only a minimum growth inhibitory effect but the inactivation of mTOR blocked protein synthesis, resulting in a reduction of TS, to keep 5-FU in an active form. In HER2 overexpressing cancer cells, PI3K signalling which is downstream of HER2, is known to promote cell survival through activation of AKT/mTOR-related pathways, and the high expression of HER2 is also recognised to induce downstream signal, and the combination of 5-FU with Rap may be also be effective through an associated mechanism.

In conclusion, the recent molecular targeting chemotherapeutic agents for HER2 are expected to block the downstream signal, and the combination of 5-FU with Rap may be also be effective through an associated mechanism, even for highly HER2-expressing breast cancer cells.

References

29. Hosono et al: Combination Therapy of 5-FU with Rapamycin in Human Breast Cancer Cell Lines

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