

## The Tyrosine Kinase Inhibitor AG490 Inhibits Growth of Cancer Cells and Activates ERK in LS174T and HT-29 Cells

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**Abstract.** *Background:* The activity of tyrosine kinases, although strictly regulated in normal cells, is often disturbed in cancer cells. The inhibition of a tyrosine kinase could be a target for treating cancer. *Materials and Methods:* The colon cancer cell lines LS174T and HT-29 and the lung cancer cell line NCI-H292 were used. The cells were incubated with 100  $\mu$ M of the tyrosine kinase inhibitor AG490 for 1-3 days and were examined for growth. Extracellular signal-regulated kinase (ERK) activation was detected by anti-phospho ERK antibodies. The cell cycle was analyzed by flow cytometry. *Results:* AG490 inhibited the growth of LS174T, HT-29 and NCI-H292 cells without inducing apoptosis. Short-term treatment with AG490 activated ERK and p38 MAPK in the LS174T and HT-29 cells, but not in NCI-H292 cells. ERK activation, however, was unrelated to the growth inhibition in LS174T cells, because the inhibition persisted even after the prevention of ERK activation. *Conclusion:* AG490 inhibits the growth of some cancer cells and activates ERK in LS174T and HT-29 cells. ERK activation is unrelated to growth inhibition.

In mammalian cells, many cell surface receptors involved in cell growth have tyrosine kinases in their intracellular domains. Binding signals with the ligands activate tyrosine kinases and stimulate cascades of intracellular signaling proteins. Successive signals activate the cascade of MAP kinase, which plays important roles in cell proliferation and differentiation. The superfamily of MAP kinases includes the extracellular signal-regulated kinase (ERK) family, the p38 MAPK family and the stress-activated protein kinase or c-Jun NH<sub>2</sub>-terminal kinase family (1, 2). The activation of ERK1/2 is usually transient in response to extracellular signals.

The activity of protein kinases is strictly regulated in the physiological conditions of normal cells but is, however, often disturbed in many cancer cells. The constitutive

activation of ERK1/2 has been detected in some cancer cell lines, which are derived from the pancreas, colon, lung, ovary and kidney, because of over activation of tyrosine kinases (3). Therefore, the inhibition of a tyrosine kinase could be a target in the treatment of cancer (4). The tyrosine kinase inhibitor AG490 is often used for the inhibition of JAK activity (5-7). JAK proteins, which are associated with cytokine receptors, phosphorylate STAT proteins. The activated STAT dimer stimulates the transcription of specific genes in the nucleus. We found that AG490 inhibited the growth of the LS174T and HT-29 colon cancer cell lines and of NCI-H292, a lung cancer cell line, without inducing apoptosis. Interestingly, the tyrosine kinase inhibitor AG490 activated ERK and p38 MAPK in the colon cancer cells after a short-term treatment. ERK activation, however, was unrelated to the growth inhibition of LS174T cells.

### Materials and Methods

*Reagents.* The cell culture media, RPMI-1640, McCoy's 5A and minimum essential medium eagle (MEM), were obtained from Sigma Aldrich (Tokyo, Japan). Fetal bovine serum (FBS) was obtained from Cansera International (Ontario, Canada), non-essential amino acids solution from Invitrogen (NY, USA), and the penicillin-streptomycin and trypsin-EDTA solutions from Gibco Oriental (Tokyo, Japan). AG490, U0126 and JAK Inhibitor I were obtained from Wako Co. (Osaka, Japan) and the EGF receptor inhibitor, AG1478, was obtained from Merck (Tokyo, Japan).

*Antibodies.* Anti-phospho p44/p42 MAP kinase (ERK1/2, Thr 202/Tyr 204) E10 monoclonal antibody (Cell Signaling Technology, MA, USA), anti-phospho STAT6 (Tyr 641) polyclonal antibody (Cell Signaling Technology), anti-phospho JAK2 (Tyr 1007/Tyr 1008) polyclonal antibody (Santa Cruz, CA, USA), anti-phospho p38 MAPK (Thr 180/Tyr 182) polyclonal antibody (Research and Diagnostic Systems, Tokyo, Japan), anti- $\beta$ -actin monoclonal antibody (Sigma Aldrich), goat anti-mouse IgG antibody conjugated with horseradish peroxidase (HRP, Promega, WI, USA) and goat anti-rabbit IgG antibody conjugated with HRP (Promega) were used in this investigation.

*Cell culture.* The human colon adenocarcinoma lines, LS174T and HT-29, and the human lung mucoepidermoid carcinoma line, NCI-H292, were purchased from the American Type Culture Collection

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*Key Words:* AG490, ERK, tyrosine kinase, colon cancer, cell cycle.

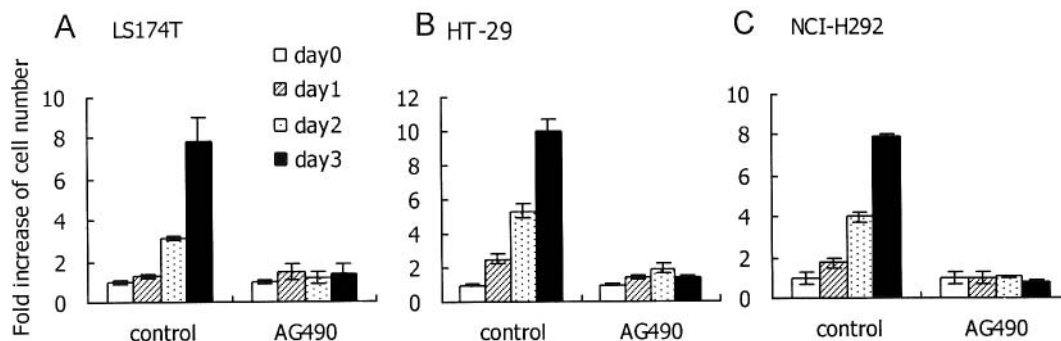


Figure 1. AG490 inhibited proliferation of LS174T (A), HT-29 (B) and NCI-H292 (C) cells. The cells were cultured with AG490 (100 μM) or 1% DMSO medium (control). The fold increase of cell growth against initial cell number (day 0) is shown.

(MD, USA). The LS174T cells were cultured in 10% FBS-MEM and 1% non-essential amino acids with 100 U/ml penicillin and 100 μg/ml streptomycin, in a 5% CO<sub>2</sub> incubator. The HT-29 cells were cultured in 10% FBS-McCOY's 5A modified medium and 1% non-essential amino acids with 100 U/ml penicillin and 100 μg/ml streptomycin. The NCI-H292 cells were cultured in 10% FBS-RPMI-1640 with 100 U/ml penicillin and 100 μg/ml streptomycin. The adherent cells were subcultured with trypsin-EDTA every 3-4 days.

**Cell proliferation assay.** The cells (2x10<sup>3</sup>) were cultured in 0.1 ml medium in a 96-well plate (Sumilon, Tokyo, Japan) overnight. After the medium had been removed, the cells were cultured in 0.1 ml of new medium containing AG490 or an appropriate control for 1, 2 and 3 days. On the day indicated, the medium was removed and the cells were incubated for 4 h with 0.1 ml of the new medium containing 10 μl of the reagent of the Cell Counting Kit-8 (Dojin, Tokyo, Japan). Absorbance at 450 nm was measured by a microplate reader Model 550 (Bio-Rad, Tokyo, Japan).

**Western blotting.** Cells (1x10<sup>5</sup>) in 0.1 ml of medium and cells (2x10<sup>5</sup>) in 0.2 ml of medium were cultured in a 96-well plate and a 48-well plate, respectively, overnight. After the media had been removed, the cells were treated with 0.1 ml of medium containing the inhibitor or appropriate control for the time indicated. The treated cells were dissolved in 0.05 ml or 0.1 ml of Laemmli's sample buffer and applied to Western blotting, by the method previously described (8). Briefly, the proteins on the SDS-polyacrylamide gel were transferred to a nitrocellulose membrane. The membrane was then immersed in a blocking buffer and incubated with the first antibodies (1:2000). Subsequently, the membrane was washed and incubated with the second antibodies conjugated with HRP (1:2000). The enzyme reaction was detected using the ECL Western Blotting Detection Reagent (Amersham Biosciences, Buckinghamshire, UK) and an LAS-1000 Plus image analyzer (Fujifilm, Tokyo, Japan).

**Detection of apoptosis.** The cells (8x10<sup>3</sup>) in 0.4 ml medium were cultured in a 24-well plate overnight. After the medium had been removed, the cells were treated with 0.4 ml of new medium containing AG490 (100 μM), 1% DMSO control or hydrogen peroxide (30 mM) for 48 h. Genomic DNA was extracted from the cells with 0.15 M NaCl, 0.1 M EDTA and 2.3% SDS. The

extracted DNA was treated with RNase A (100 μg/ml, Nippon Gene, Tokyo, Japan) and was then applied to 1.5% agarose gel electrophoresis with ethidium bromide. The other portion of the cells, treated as above, was fixed with 10% formalin (Mildform 10N, Wako) and stained with fluorescent, Hoechst 33342 (10 μg/ml, Invitrogen) for 15 min. The stained cells were observed by a fluorescence microscope, BX51 (Olympus, Tokyo, Japan), with excitation at 365 nm and emission at 400 nm.

**Flow cytometry.** The cells (2x10<sup>5</sup>) in 0.4 ml complete medium (10% FBS) were cultured for 1 day in a 24-well plate and were then cultured with incomplete medium (0.5% FBS) for 2 days. After the incomplete medium had been removed, the cells were cultured again with fresh complete medium containing AG490 (100 μM) or 1% DMSO control for 24 h and 48 h. Following removal of the medium, the cells were treated with 0.1% Triton X-100 in PBS for 5 min. The cell nuclei were collected, treated with RNase A (0.01 mg/ml, Wako) and stained with propidium iodide (0.04 mg/ml, Sigma Aldrich) and the stained nuclei were analyzed by a flow cytometer, FACSCalibur, with the software CellQuest (Becton Dickinson, NJ, USA). For each sample, data were obtained through 2 gates, FSC vs. SSC and FL2W vs. FL2A, to exclude emission of binucleated cells. At least 10,000 nuclei were counted for each sample.

## Results

AG490 at concentrations of 10, 50 and 100 μM inhibited the growth of LS174T cells by 8, 40 and 51%, respectively, on day 2 of culture. AG490 was used at a concentration of 100 μM thereafter unless indicated. The growth inhibition was clear in the LS174T, HT-29 and NCI-H292 cells on day 3 of culture (Figure 1). Whether the cell growth inhibition observed was induced by apoptosis was then examined. Treatment of LS174T cells with AG490 showed neither DNA fragmentation nor nuclear condensation, which are typically observed in apoptotic cells (Figure 2). No induction of apoptosis with AG490 was detected in either the HT-29 or NCI-H292 cells. The 3 cancer cell lines were treated with AG490 for 2 days and cultured again without AG490. After the AG490 had been removed, the number of cells increased

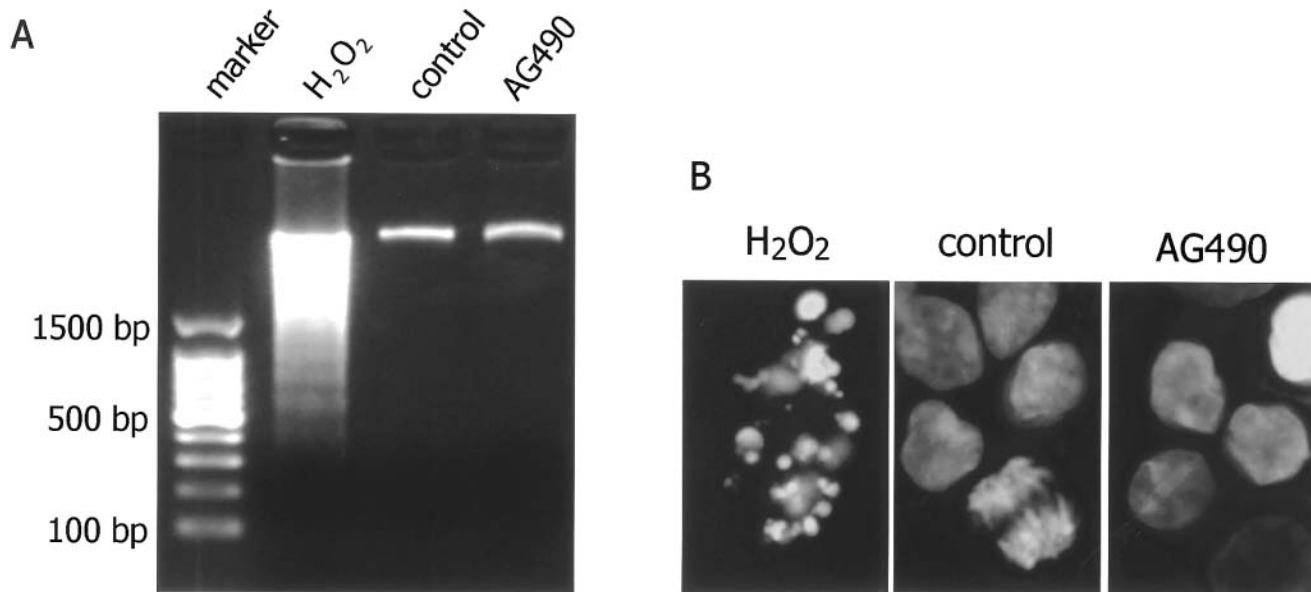


Figure 2. AG490 did not induce apoptosis in LS174T cells. A) The cells were cultured with AG490 (100  $\mu$ M), 1% DMSO medium (control) or  $H_2O_2$  (positive control) for 2 days. The genomic DNA was extracted and applied to agarose gel electrophoresis. B) The cells were stained with Hoechst 33342 and observed by a fluorescence microscope.

by about 2.5-fold on day 2 of the new culture and by 4.5-fold on day 4, suggesting that AG490 inhibited the growth of the cancer cells but did not kill them.

The activation of ERK1/2 is usually induced within a few minutes in response to extracellular signals and is rapidly diminished by dephosphorylation. Although the duration of ERK1/2 activation varies from minutes to hours depending on the type of signals, cell proliferation or differentiation begins several hours after activation. We found that the treatment of LS174T cells with more than 100  $\mu$ M of AG490 activated ERK and p38 MAPK (Figure 3A). The activation of ERK and p38 MAPK continued for at least 45 min (Figure 3B), but no significant activation of ERK or p38 MAPK was observed in LS174T cells upon treatment with other tyrosine kinase inhibitors, such as the JAK Inhibitor I (5-250 nM, 2.5-60 min) or the EGF receptor inhibitor AG1478 (0.01-10  $\mu$ M, 5-30 min).

AG490 is often used to inhibit JAK2 activity. The LS174T cells were treated with AG490 for 4 h and stimulated with IL-13. AG490 was found to inhibit the phosphorylation of STAT6 by IL-13 in LS174T cells (data not shown), suggesting that the treatment of cells with AG490 inhibits JAK2 activity, as reported. However, when the LS174T cells were treated simultaneously with AG490 and IL-13 for 5 to 45 min, AG490 did not inhibit STAT6 activation (Figure 4). These experiments show that AG490 can inhibit the JAK/STAT pathway after 4 h of incubation, but also activates ERK and p38 MAPK in a short-term

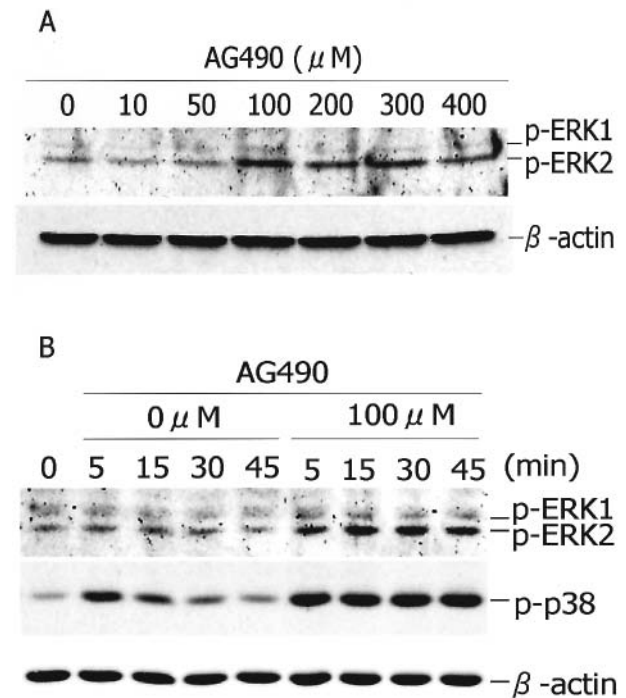


Figure 3. Short-term treatment of LS174T cells with AG490 activated ERK and p38 MAPK. A) The cells were treated with different concentrations of AG490 for 15 min. B) The cells were incubated with AG490 (100  $\mu$ M) or 1% DMSO medium (0  $\mu$ M) for the time indicated. 0 min represents cells incubated with culture medium without DMSO. The cells were then solubilized and subjected to Western blotting.

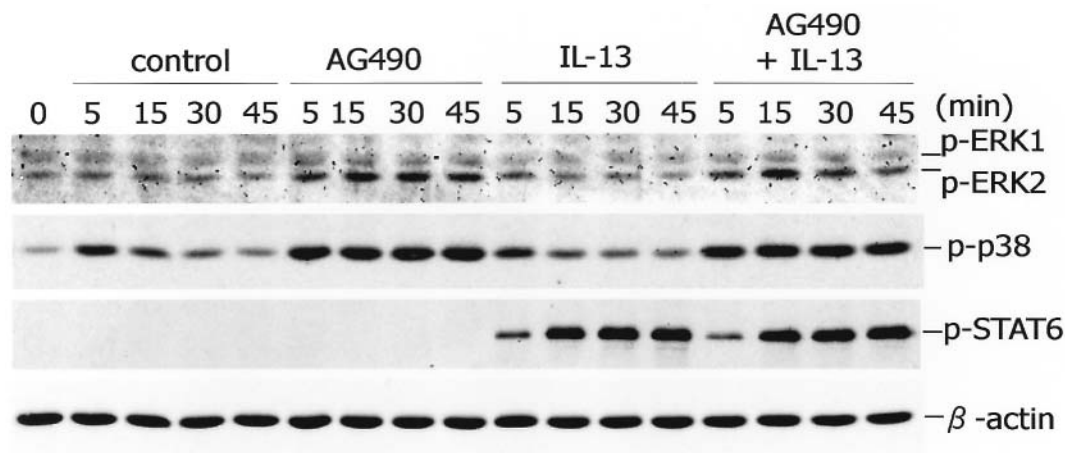


Figure 4. The simultaneous addition of AG490 and IL-13 activated ERK, p38 MAPK and STAT6 in LS174T cells. The cells were treated with AG490 (100  $\mu$ M), IL-13 (10 ng/ml) or a mixture of the two for the time indicated.

incubation (5-45 min), without inhibiting the JAK/STAT pathway in LS174T cells. ERK and p38 MAPK activation was also observed in the HT-29 cells upon incubation with AG490 for 5 min to 2 h. The results of incubation from only 30 min to 2 h are illustrated in Figure 5A. On the contrary, no significant activation of ERK or p38 MAPK was observed in NCI-H292 cells upon incubation with AG490 (100 and 200  $\mu$ M) for 5 min to 2 h. The results of only 5 to 12.5 min of incubation with 100  $\mu$ M of AG490 are shown in Figure 5B.

In order to verify whether the cell growth inhibition was correlated with ERK activation in the AG490 treatment, the effects of the MEK inhibitor U0126 were examined. The LS174T cells were pretreated with U0126 for 4 h and cultured with AG490 for 1 to 3 days. The U0126 treatment showed no change in cell growth inhibition with AG490, although ERK activation with AG490 was eliminated by U0126 treatment (Figure 6), indicating that AG490 inhibited cell growth independently of ERK activation in the LS174T cells.

The effect of AG490 on the cell cycle was analyzed by flow cytometry in the nuclei separated from the cancer cells. The starved LS174T cells showed 81.8%, 7.6% and 10.6% of G0/G1-, S- and G2/M-phases, respectively. When the starved cells were cultured in the complete medium for 24 h, the G0/G1-phase decreased to 73.7% and the S-phase increased. However, the decrease in the G0/G1-phase was not detected in the cells treated with AG490 (Figure 7A).

Similar results were observed in the NCI-H292 cells, but not in the HT-29 cells (Figure 7B and C). The cell cycle analysis revealed that AG490 caused cell cycle arrest at the G0/G1- or S-phases in the LS174T and NCI-H292 cells on

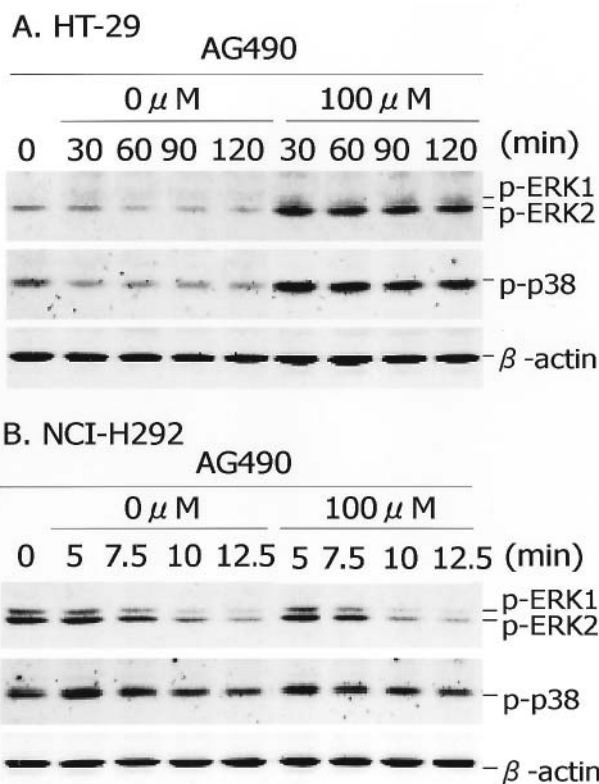


Figure 5. Treatment with AG490 activated ERK and p38 MAPK in HT-29 cells (A) but not in NCI-H292 cells (B). The cells were treated with AG490 (100  $\mu$ M) or 1% DMSO medium (0  $\mu$ M) for the time indicated. 0 min represents those cells incubated in culture medium without DMSO.

day 1 of culture. No significant change in G0/G1-phase was observed in the HT-29 cells.



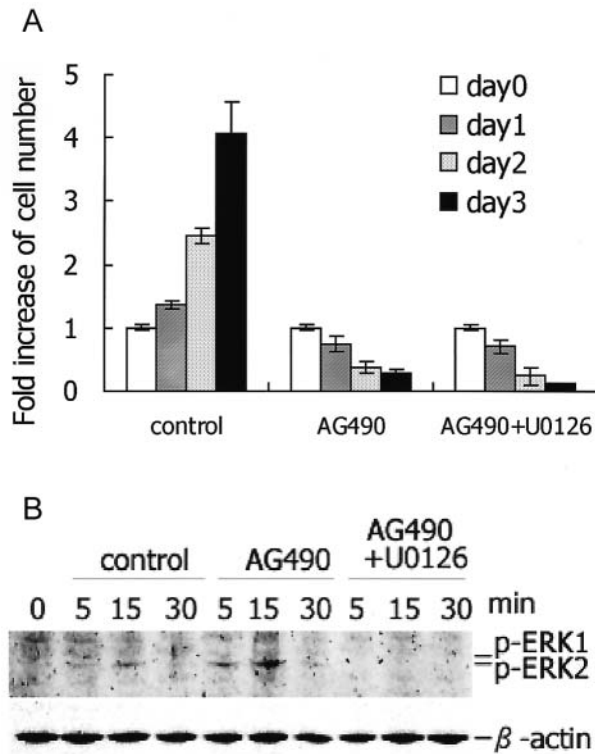


Figure 6. The inhibitory effect of AG490 on LS174T cell growth was unrelated to ERK activation. A) The cells were pretreated with a MEK inhibitor, U0126 (30  $\mu$ M), or 2.3% DMSO medium (control) for 4 h and were then cultured with AG490 (100  $\mu$ M) or the control medium for the days indicated. Each group of control, AG490 and AG490+U0126 contained 2.3% DMSO in the culture medium. B) The cells corresponding to the above were solubilized at the time indicated after the addition of AG490 or the control and were subjected to Western blotting. 0 min represents those cells incubated with culture medium without DMSO.

## Discussion

The tyrosine kinase inhibitor tyrphostins, including AG490, inhibit cell growth *via* cell cycle arrest at late G1- and S-phases in the DHER 14 fibroblast cell line in which the EGF receptors are overexpressed (9). AG490 also inhibits G1-S transition in BALB/c-3T3 cells stimulated with platelet-derived growth factor or the adenovirus inserted human *E2F-1* gene (10). In our experiments, AG490 inhibited the growth of LS174T, HT-29 and NCI-H292 cells without induction of apoptosis. The treated cells proliferated again upon removal of AG490. These results indicate that AG490 arrested the cell cycle but did not kill the cells, as reported previously (9, 10). AG490 is often used as a JAK2 inhibitor to inhibit the JAK/STAT signaling pathway, but it can also inhibit cyclin-dependent kinases, the autophosphorylation of the epidermal growth factor receptor and c-Src activity (11). The cell cycle arrest

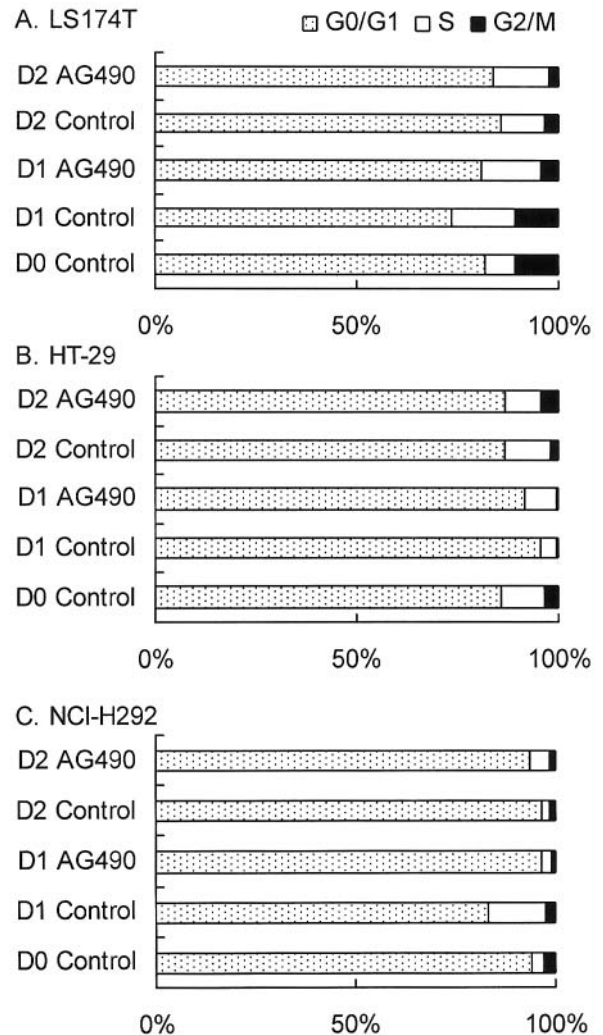


Figure 7. AG490 changes the cell cycle in the cancer cells. The cells were starved and cultured again with medium containing AG490 (100  $\mu$ M) or the control. The separated cell nuclei were analyzed by flow cytometry. The percent phases of G0/G1 (dotted bars), S (white bars) and G2/M (black bars) are shown in LS174T (A), HT-29 (B) and NCI-H292 (C). The left indices show the starved cells (D0 Control), the cells treated for 24 h with complete medium control (D1 Control) or AG490 (D1 AG490), and those cells treated for 48 h with complete medium control (D2 Control) or AG490 (D2 AG490).

observed in the colon and lung cancer cells might be induced by the inhibition of cyclin-dependent kinases with AG490.

AG490 inhibited STAT6 phosphorylation after a 4-h treatment, but the short-term treatment of cells with AG490 activated ERK phosphorylation in the colon cancer cells. The ERK activation by AG490, however, was not correlated with cell growth arrest. It was reported that AG490 inhibits the angiotensin II-dependent activation of ERK by a

JAK-independent mechanism (11), but this is the first report, to our knowledge, of the activation of ERK by AG490. Since AG490 is usually added to the cell culture for more than a few hours to inhibit the JAK/STAT signaling pathway, the function of ERK activation following a short-term incubation with AG490 has not been studied.

A typical signaling pathway of the MAP kinase cascade is activated through receptor phosphorylation and Ras and Raf activation. MAP kinase signaling induces a wide range of different biological responses in different cell types. The Src tyrosine kinase inhibitor PP2 is known to enhance Ras-independent activation of the Raf-1 kinase (12). Activation of the Raf kinase may induce various changes in the cells, such as proliferation, differentiation, apoptosis or cell cycle change (13). Therefore, the tyrosine kinase inhibitor AG490 might activate ERK through the Ras-independent activation of Raf kinase. We have not determined the actual target tyrosine kinases and the importance of ERK activation by AG490 in the colon cancer cells. Our results showed that AG490 is not only a JAK2 inhibitor, but can also activate ERK under some conditions.

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