Simultaneous Inhibition of Mitogen-activated Protein Kinase and Phosphatidylinositol 3-Kinase Pathways Augment the Sensitivity to Actinomycin D in Ewing Sarcoma

INTRODUCTION
EWS/Fli-1 fusion is a product of translocation t(11;22) (q24;q12), which encodes a transcriptional activator and promotes cellular transformation. More than 85% of ESFTs retain this chimeric fusion gene, which plays certain roles in the regulation of telomerase activity, tumor angiogenesis, and phospholipase D (PLD) expression. Although substantial studies have reported reduced tumorigenicity and clonogenicity by antagonizing EWS fusion gene, its detailed biological targets remain unknown. Our previous studies have indicated that EWS/Fli-1 gene regulated PLD2 expression, which is implicated in the activation of proliferation via mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway and the cell survival via phosphatidylinositol 3-kinase (PI3K)/Akt pathway.

To investigate the role of EWS/Fli-1 in ActD resistance, the present study examined whether transfection of Ewing sarcoma TC-135 cells with short interfering RNAs (siRNAs) for EWS/Fli-1 affects the antitumor effect of ActD. We then investigated how antitumor effect of ActD is influenced by modulating MEK/ERK and PI3K/Akt pathways which are downstream targets of EWS/Fli-1 using their inhibitors in Ewing sarcoma cells and in the xenograft mouse model.

METHODS
Cytotoxicity was measured by WST-8 assay (Wako Junyaku, Osaka, Japan). Apoptotic cells were stained with Fluorochrome Inhibitor of Caspases (FLICA) Apoptosis Detection Kit (Immunochemistry Technologies, Bloomington, MN) and examined by fluorescence microscope. Expression protein was analyzed by western blotting. Caspase activity was determined by Caspase-Glo assay (Promega, Madison, WI). The sequences of siEF are 5’-AGCAGAACCCUUCUUAUGACUU and 5’-UUAGAGGCUUGGAAAGAUUCG-3’. The cells were cotransfected with silencer Cy3 labeled negative control siRNA #1 (A5180) and siEF, to determine transfection efficiencies. A nude mouse tumor xenograft model was established for in vivo experiments. Male BALB/c athymic (nu/nu) nude mice (5-6 weeks old) were obtained from Japan SLC. Mice were housed in the animal facility of the Division of Animal Experiment, Life Science Research Center, Gifu University. All animal work was conducted under protocols approved by the Gifu University Animal Experiment Committee. Unpaired t test was used for analyzing the two pairs of data in the experiments. ANOVA was used for comparing the data with more than two treatments in the experiments. A P value < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION
ActD induced dose- and time-dependent cell death in TC-135 cells. ActD treated TC-135 cells were then transfected with FLICA Apoptosis Detection Kit and subsequently labeled with propidium iodide (PI) and Hoechst stain. ActD treated cells showed significant increase of FLICA-positive cell fraction compared to that of vehicle treated cells. Furthermore, FLICA-positive cells were compared with PI-stained cells to investigate the overall percentage of apoptotic cells in total cell death. As a result, 94.3 % of PI-stained ActD treated cells were FLICA-positive suggesting that over 90 % of ActD-induced TC-135 cell deaths are caspase-dependent apoptosis. Caspase 3/7 Glo assay and Western blotting demonstrated that ActD induced dose- and time-dependent increase of caspase 3/7 activity and PARP cleavage in TC-135 cells.

To understand the role of EWS/Fli-1 as pertains to apoptosis induction, sIEF and siCONTROL were transfected into Ewing sarcoma TC-135 cells. Western blotting showed significant downregulation of EWS/Fli-1 protein expression of siEF transfected cells. Furthermore, siEF-transfected cells incubated with ActD displayed significant enhancement of PARP cleavage. These results showed that knockdown of EWS/Fli-1 fusion by siRNA resulted in enhancement of ActD induced apoptosis, therefore implying that EWS/Fli-1 and presumably its downstream targets play important roles in acquiring resistance to ActD-induced apoptosis.

Several studies have demonstrated that EWS/Fli-1 fusion gene is associated with MEK/ERK or PI3K/Akt pathways. Western blot showed that ActD treatment triggers the activations of both ERK and Akt in TC-135 cells. Preincubation with 5 µM U0126 (MEK inhibitor) or 10 µM LY294002 (PI3K inhibitor) made the cells more susceptible to ActD treatment with a decrease of IC50 from 18.91 to 5.79 or 4.83 ng/ml, respectively. The combined preincubation with both U0126 and LY294002 rendered the cells even more vulnerable to ActD treatment with a decrease of IC50 from 18.91 to 3.34 ng/ml. These results are considered very important in suggesting that even lower concentration of ActD can achieve the higher anti-tumor effect by combining these inhibitors. The Western blot demonstrated the specific inhibition of P-Akt and P-ERK by 10 µM LY294002 and 5 µM U0126, respectively. ActD-induced PARP cleavage was enhanced mainly by the inhibition of PI3K/Akt pathway but not MEK/ERK pathway. These results indicate that both MEK/ERK and PI3K/Akt pathways are involved in the cell survival, although through distinct mechanisms.

A nude mouse tumor xenograft model was used to evaluate the anti-tumor effect of ActD, LY2940002, U0126, and their combinations in vivo. When the tumors reached a volume of 50 - 70 mm³, animals were randomized into four groups (n=6). 1) ActD (0.045 mg/kg), 2) ActD (0.225 mg/kg), 3) LY294002 (20 mg/kg) and U0126 (4 mg/kg), or 4) combination of ActD (0.045 mg/kg) with LY2940002 (20 mg/kg) and U0126 (4 mg/kg). The reagents were administered by single weekly ip injections and tumor growth was measured every 3 days for over a period of up to four weeks. Group 4 showed an anti-tumor effect comparable to that of a higher dose (0.225 mg/kg) of ActD (group 2). Importantly, we have demonstrated that a combined therapy involving U0126 and LY2940002 significantly enhanced or maintained the anti-tumor effect of ActD, without an increase in the drug dose and thus its toxicity. No mice deaths, loss of body weight, or unusual behavior occurred as a result of combination therapy using LY2940002 and U0126. These findings may indicate the practical possibility of the use of MEK/ERK and PI3K/Akt pathway inhibitors in combination with conventional chemotherapeutics for the treatment of ESFT patients.

ACKNOWLEDGEMENTS
The authors thank Dr. T. J. Triche (University of Southern California, Los Angeles, CA) for providing the Ewing sarcoma cells and Dr. Yoshiko Banno (department of Cell Signaling, Gifu University) for useful discussion.