Imatinib mesylate (STI571) enhances amrubicin-induced cytotoxic activity through inhibition of the phosphatidylinositol 3-kinase/Akt pathway in small cell lung cancer cells

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Abstract. Small cell lung cancer (SCLC) is characterized by autocrine mechanisms. Stem cell factor (SCF) and its receptor c-kit can activate Akt and extracellular signal-regulated kinase (Erk) pathways. Imatinib mesylate (STI571) can inhibit c-kit tyrosine kinase activity, but clinical trials have resulted in failure. We investigated the possibility of SCF/c-kit-targeted therapy against SCLC. Using c-kit-positive SCLC cells (H209 and H69 cells) and SCF as a model of the autocrine mechanisms, the effects of SCF, LY294002, PD98059 or STI571 on Akt and Erk were assessed by Western blot analysis. The cell growth inhibitions of cisplatin, etoposide irinotecan and amrubicin (AMR) with or without SCF, LY294002, PD98059 or STI571 were evaluated by MTT assay. Treatment with SCF activated Akt and Erk and the activations were inhibited by STI571 in H209 but not in H69 cells. LY294002 and PD98059 inhibited SCF-induced Akt and Erk activation in H209 cells, respectively. STI571 alone did not exert growth inhibition in the SCF-treated cells. In H209 cells, SCF decreased the cytotoxicity of AMR, but not of other drugs. In H69 cells, SCF did not affect sensitivity to any drugs. LY294002 but not PD98059 restored or enhanced AMR-sensitivity in SCF-treated H209 or untreated H69 cells, respectively. STI571 restored the AMR-sensitivity of SCF-treated H209 cells to the basal level. If the SCF/c-kit contributes to Akt activation *in vivo*, the combination of STI571 and AMR may be effective against SCLC. Additionally, using a combination of AKT inhibitors and AMR may be a promising treatment in the future.

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Introduction

Small cell lung cancer (SCLC) is a highly aggressive neoplasm characterized by a high growth fraction, short doubling time and high rate of metastasis (1), but is sensitive to chemotherapy (2). Systemic chemotherapy prolongs the survival of SCLC patients (3), and cisplatin (CDDP), etoposide (VP-16), topoisomerase I inhibitors [irinotecan (CPT-11) and topotecan] (4,5) and amrubicin (AMR) (6) (a totally synthetic 9-amino-anthracycline) are used in regular clinical practice in Japan. Among these chemotherapeutic agents, the combination chemotherapy of CDDP and VP-16 is considered the standard first line chemotherapeutic regimen worldwide (7). Despite high response rates with these first line CDDP-based chemotherapies, most patients eventually experience disease progression. Accordingly, novel chemotherapeutic regimens based on new conceptions are needed for treating SCLC patients.

SCLC is, per se, characterized by several autocrine growth mechanisms including stem cell factor (SCF) and its receptor c-kit, and c-kit overexpression is found in up to 70% of SCLC (8). In view of c-kit signal transduction, an SCF/c-kit can activate several intracellular pathways including phosphatidylinositol 3-kinase (PI3K)/Akt and mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (MEK/Erk) pathways (9).

Imatinib mesylate (STI571) was developed as a Bcr-Abl inhibitor and has become a standard therapeutic drug for chronic myelogenous leukemia (10). STI571 can also inhibit c-kit tyrosine kinase activity and has high efficacy in chemoresistant c-kit-positive gastrointestinal stromal tumors (11).

Taking these previous findings into consideration, researchers have evaluated the efficacy of STI571 in the treatment of SCLC. Two phase II studies of STI571 alone in unselected (12) or c-kit-positive (13) SCLC patients failed to show its efficacy. Furthermore, a phase I clinical trial using STI571, CDDP and CPT-11 revealed no combination effects between STI571 and these cytotoxic agents (14).

However, if the autocrine mechanism of SCF is functioning in SCLC *in vivo*, it is still possible that the SCF/c-kit-targeted therapy may be effective in the treatment of SCLC. Thus, we

investigated the effect of SCF and STI571 on SCLC cell growth and the combination effects of STI571 with other chemotherapeutic agents used in regular clinical practice using c-kit-positive SCLC cells.

Materials and methods

Chemicals and reagents. CDDP (a gift from Nippon Kayaku, Co. Ltd, Tokyo, Japan) and AMR (a gift from Dainippon Sumitomo Pharma Co. Ltd, Osaka, Japan) were dissolved in distilled water and stored at -20˚C after filtration. VP-16 (Wako Pure Chemical Industries, Ltd, Osaka, Japan) and CPT-11 (a gift from Daiichi Pharmaceutical, Co. Ltd., Tokyo, Japan) were dissolved in dimethylsulfoxide and stored at -20˚C. Recombinant human SCF (a gift from Kirin Brewery Co. Ltd, Tokyo, Japan) was stored at 4˚C. 2'-Amino-3'-methoxyflavone (PD98059) (Calbiochem, San Diego, CA, USA) and 2-(4-Morpholinyl)-8-phenyl-4H-l-benzopyran-4-one (LY294002) (Sigma-Aldrich Japan, Tokyo, Japan) was dissolved in dimethylsulfoxide and stored at -20˚C. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Chemical Co., St. Louis, MO, USA) was dissolved in phosphate-buffered saline (PBS) and stored at -20˚C.

Cells. H209 and H69 human SCLC cell lines were provided by Dr A.F. Gazdar and Dr H. Oie (NCI-Navy Medical Oncology Branch, NIH, Bethesda, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics. The cells were grown in a humidified atmosphere of 5% CO_2-95% air.

MTT assay. The effects of SCF on the cell proliferation in the H209 and H69 cells were measured by MTT assay using 96-well flat bottom multiplates (Nalge Nunc International K.K., Denmark). The cells were counted with a hematocytometer and $5x10^4$ cells were treated with or without 100 ng/ml of SCF for 24, 48 or 72 h. Next, 20 μg of MTT in 10 μl PBS was added to each well and incubation was performed for an additional 4 h. Thereafter, 100 μl of 0.04 N HCl in 2-propanol was added and incubated overnight in order to solubilize the MTT formazan crystals. The absorbance of each well was measured at a 570-nm wavelength (reference 650 nm) using a scanning multiwell spectrophotometer (MPR A4i, Tosoh Co, Tokyo, Japan). The cytotoxic activity of AMR in the H209 and H69 cells was evaluated by MTT assay in the same way as described above using 100 ng/ml of SCF and 10 μM of STI571.

Cell lysis, immunoprecipitation and Western blot analysis. The cells were lysed in a modified radioimmune precipitation buffer (1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, 100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 2 mM EDTA, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 40 mM ß-glycerophosphate and 2 mM Na_3VO_4) and insoluble material was removed by centrifugation. The protein concentration was determined by means of a Bio-Rad Protein assay (Bio-Rad, CA), and lysates containing 50 μg of total cellular protein or immunoprecipitates with indicated antibodies were analyzed by Western blotting after SDS-polyacrylamide gel electrophoresis and visualized by
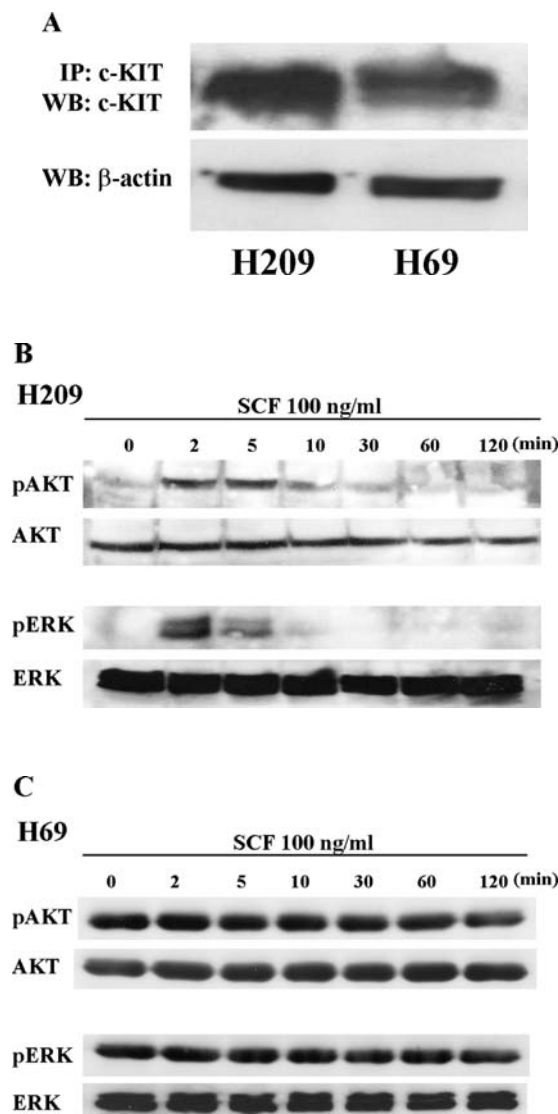

Figure 1. Effects of SCF treatment on Akt and Erk activity in H209 and H69 cells. Total cellular protein (50 μg) from cell lysate was subjected to Western blot analysis with an anti-c-kit (A) as described in Materials and methods. The cells were incubated with 100 ng/ml of SCF for 2-120 min. The total cellular protein from cell lysate was subjected to Western blot analysis with anti-active Akt (pAkt) or anti-Akt (upper panel) and anti-active Erk (pErk1/2) or anti-Erk1/2 (lower panel). The results of (B) H209 cells; (C) H69 cells.

enhanced chemiluminescence detection (Amersham Pharmacia Biotech) using donkey anti-rabbit IgGs coupled to horseradish peroxidase as a secondary antibody (Amersham Pharmacia Biotech). The anti-Erk1/2 and anti-active Erk antibodies were purchased from Promega Corporation. The anti-Akt and anti-active Akt antibodies were purchased from Cell Signaling Technology. The anti-c-kit antibody was purchased from Dako Cytomation.

Results

Effects of SCF treatment on Akt and Erk activity in H209 and H69 cells. The effects of the SCF-treatment on Akt and Erk activation in the H209 and H69 cell lines were investigated. First, we confirmed the expression of c-kit protein in the H209 and H69 cells (Fig. 1A). Both cells were treated with 100 ng/ml
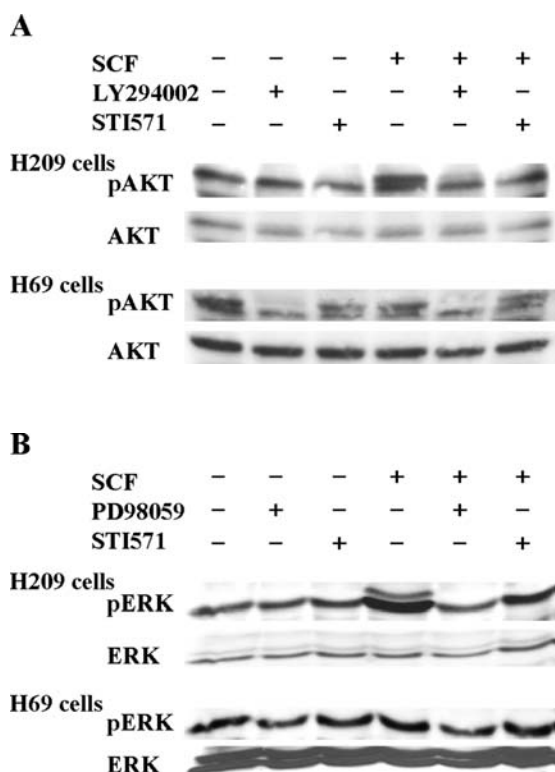
A

B

Figure 2. Effects of STI571 on the activity of Akt and Erk in H209 and H69 cells. (A) LY294002 (25 μM) and 10 μM of STI571 were added for 2 min, just before the cells were incubated with 100 ng/ml of SCF. Total cellular protein (50 μg) from cell lysate was subjected to Western blot analysis with anti-active Akt (pAkt) or anti-Akt as described in Materials and methods (A). In the same way, the cells were treated with 50 μM of PD98059 and 10 μM of STI571 for 2 min, just before the incubation with SCF in (B). The total cellular protein from cell lysate was subjected to Western blot analysis with anti-active Erk (pErk1/2) or anti-Erk1/2 as described in Materials and methods (B).
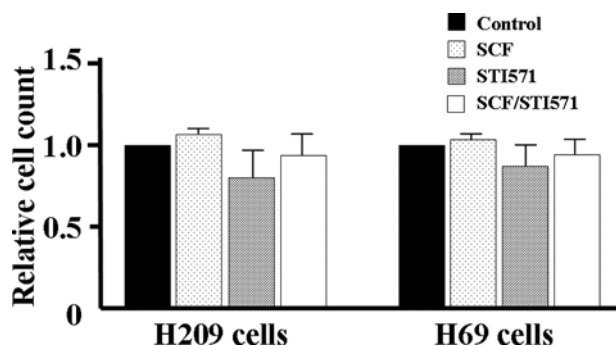

Figure 3. Effects of SCF and STI571 on the proliferation in H209 and H69 cells. Both H209 and H69 cells were treated with or without 100 ng/ml of SCF and 10 μM of STI571 for 72 h. The cell proliferation was assessed by MTT assay and expressed as the percentage of optical density (%OD) in reference to the OD of SCF-untreated cells. Data are presented as the mean ± standard deviation of three separate experiments.

of SCF for 0-120 min. As shown, treatment with SCF clearly activated Akt and Erk at 2-5 min in the H209 cells (Fig. 1B), but did not affect Akt and Erk activity in the H69 cells (Fig. 1C).

Effects of STI571 on the activity of Akt and Erk in H209 and H69 cells. The effect of STI571 on Akt and Erk activity was evaluated with a specific inhibitor of Akt, LY294002 or Erk, PD98059 in H209 and H69 cells.

In the absence of SCF, treatment with 25 μM of LY294002 or 10 μM of STI571 did not affect the activity of Akt in H209 cells. However, treatment with 25 μM of LY294002 or 10 μM of STI571 clearly inhibited SCF-induced Akt activation in H209 cells (Fig. 2A, upper panel). In the case of H69 cells, 25 μM of LY294002 clearly inhibited the Akt activation regardless of the existence of the SCF. Ten micromolars of STI571 did not affect the activity of Akt in the H69 cells (Fig. 2A, lower panel).

Next, we investigated the effect of STI571 on the Erk activation and compared it with the effect of PD98509 on the Erk activation. In the absence of SCF, treatment with 50 μM of PD98509 or 10 μM of STI571 did not affect Erk activity in H209 cells. However, both 50 μM of PD98509 and 10 μM of STI571 inhibited SCF-induced Erk activation in H209 cells (Fig. 2B, upper panel). In the case of H69 cells, 50 μM of PD98509 showed a subtle inhibition of Erk activity regardless of the existence of SCF. Ten micromolars of STI571 did not affect Erk activity in the H69 cells (Fig. 2B, lower panel).

Effects of SCF and STI571 on the proliferation in H209 and H69 cells. To investigate whether SCF and STI571 had any effect on the cell proliferation, H209 and H69 cells were treated with 100 ng/ml of SCF, 10 μM of STI571 or a combination with the same concentration of these agents for 72 h. Although 100 ng/ml of SCF activated Akt and Erk in H209 cells, the proliferation of H209 and H69 cells did not differ with or without SCF treatment for 72 h at this concentration. The growth inhibition by 10 μM STI571 alone was marginal (~20%) compared with untreated cells in both the H209 and H69 cells. In the presence of 100 ng/ml SCF, the treatment with 10 μM of STI571 did not inhibit the cell proliferation in either of the cells (Fig. 3).

Effects of SCF treatment on cytotoxic activity of chemo-therapeutic agents in H209 and H69 cells. To investigate whether SCF had any effect on the cytotoxic activity of conventional chemotherapeutic agents used in clinical practice against SCLC, we examined cell growth inhibition by CDDP, VP-16, CPT-11 and AMR in the presence or absence of SCF in H209 and H69 cells. The cells were treated with various concentrations of these agents with or without 100 ng/ml of SCF for 72 h. The cytotoxic activity did not differ when these cells were treated with CDDP (Fig. 4A), VP-16 (Fig. 4B) and CPT-11 (Fig. 4C). Interestingly, the cytotoxic activity of AMR decreased only when H209 cells were treated with AMR in the presence of SCF. On the other hand, the cytotoxic activity of AMR did not differ when H69 were treated under the same condition (Fig. 4D).

Effects of LY294002 or PD98059 on AMR-induced cytotoxicity in SCF-treated H209 and H69 cells. To investigate whether the inhibition of Akt or Erk had any effect on the AMR-induced cytotoxic activities in the presence of SCF, H209 and H69 were exposed to various concentrations of AMR with or without 25 μM of LY294002 or 50 μM of PD98059. SCF
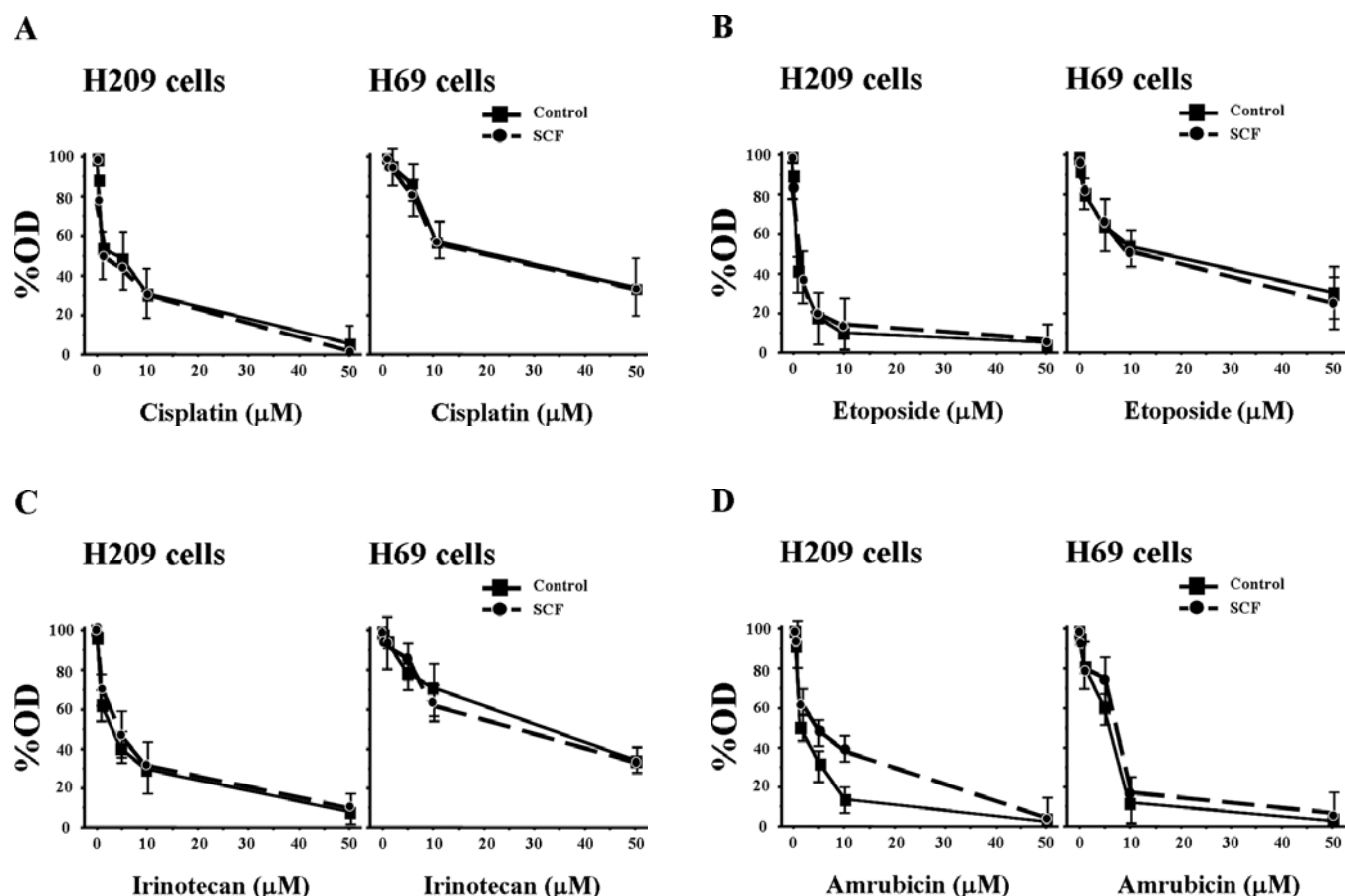
Figure 4. Effects of SCF treatment on the cytotoxic activities of cisplatin, etoposide, irinotecan and amrubicin in H209 and H69 cells. The cytotoxicity of cisplatin, etoposide, irinotecan and amrubicin was assessed by MTT assay and expressed as the percentage of optical density (%OD) in reference to the OD of untreated cells with these chemotherapeutic agents. The results are represented on the agents used with or without SCF [(A) shows on cisplatin; (B) etoposide; (C) irinotecan and (D) amrubicin]. Data are presented as the mean ± standard deviation of three separate experiments.

made the H209 cells resistant to AMR in comparison with the control and the treatment with LY294002 restored the sensitivity of H209 cells to the basal level. Although the sensitivity of H69 cells to AMR did not change in the presence of SCF, the treatment with LY294002 enhanced the sensitivity to AMR in H69 cells (Fig. 5A), whereas the treatment with PD98059 did not improve the SCF-induced AMR-resistant conditions in H209. In the H69 cells, neither SCF nor the combination of SCF and PD98059 affected the sensitivity to AMR (Fig. 5B).

Effects of SCF and STI571 on AMR-induced cytotoxicity in H209 and H69 cells. Based on these results, we examined the effects of the STI571 on the AMR-induced cytotoxicity in these two SCLC cell lines. Interestingly, when the cells were treated with 10 μM of STI571, the sensitivity of H209 cells to AMR recovered from SCF-induced resistance to the basal level. However, neither the treatment with SCF nor the combination of SCF and STI571 had any effect on the sensitivity to AMR in H69 cells (Fig. 6).

Discussion

This *in vitro* study was carried out in order to investigate the possibility of SCF/c-kit-targeted therapy in the treatment of SCLC. Using c-kit protein-positive SCLC cell lines and SCF

as a model system of the autocrine mechanisms, we examined the effects of STI571 on intracellular signaling, cell growth and conventional chemotherapeutic agent-induced cytotoxicity. STI571 as a single agent did not exert growth inhibition in SCF-treated SCLC cells. However, when combined with AMR, STI571 enhanced AMR induced cell growth inhibition in SCF-treated H209 cells.

In general, the binding of growth factor to its receptor activates intracellular signaling including the PI3K/Akt and Erk pathway (15). Although c-kit is detected in both H209 and H69 cells, the activation of Akt and Erk by SCF treatment was observed only in H209 cells. We performed all experiments in 10% FCS-containing medium. The reason that SCF did not activate Akt or Erk in H69 cells may be due to high baseline activation of these kinases by FCS. Thus, it is not surprising that the growth-promoting effect by SCF or growth-inhibitory effect by STI571 was not observed in the SCF-treated H69 cells. In addition, although SCF activated Akt and Erk, and STI571 inhibited the activation of these kinases in SCF-treated H209 cells, neither the growth-promoting effect by SCF nor the growth-inhibitory effect by STI571 was observed. H209 cells harbor loss-of-function retinoblastoma (Rb) protein (16). It was reported that Rb protein inactivation, which is observed in a majority of SCLC cases, makes Erk activation dispensable for the growth of SCLC (17). We speculate that the lack of growth-promoting effect by SCF and growth-inhibitory effect
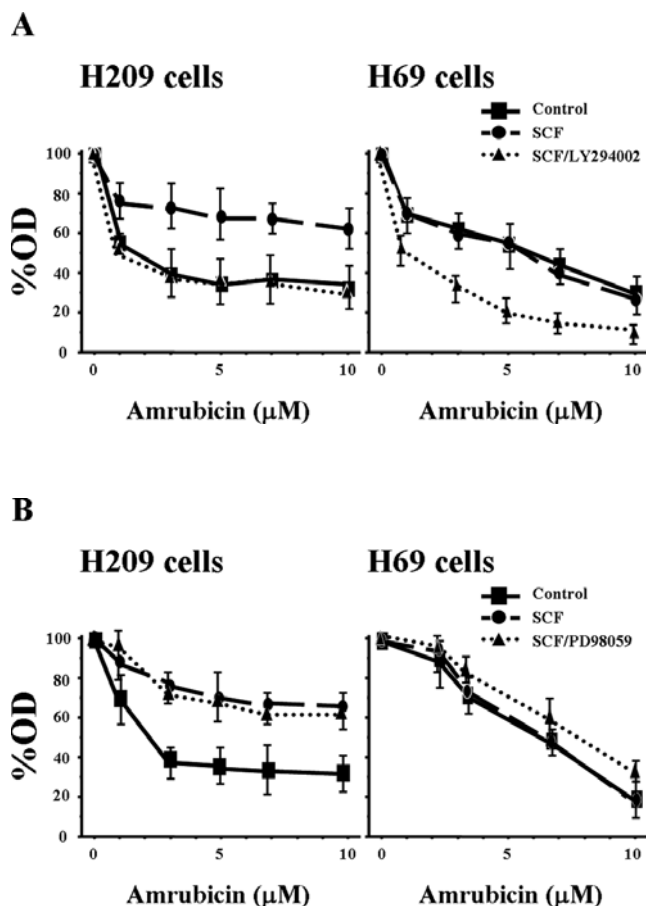
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Figure 5. Effects of SCF and LY294002 or PD98059 on amrubicin-induced cytotoxicity in H209 and H69 cells. The cytotoxicity was assessed by MTT assay and expressed as the percentage of optical density (%OD) in reference to the OD of amrubicin-untreated cells. The results are of agents used with SCF [(A) LY294002; (B) PD98059]. Data are presented as the mean ± standard deviation of three separate experiments.
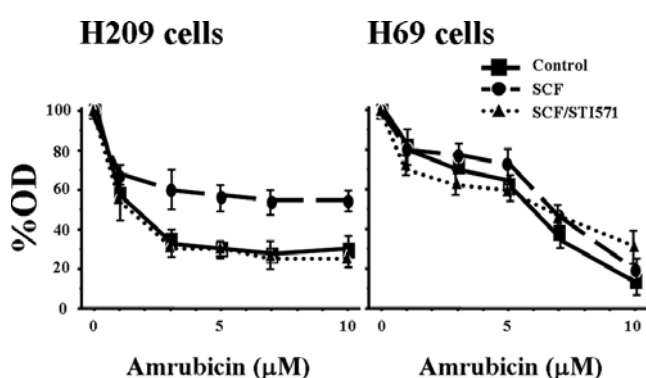

Figure 6. Effects of SCF and STI571 on amrubicin-induced cytotoxicity in H209 and H69 cells. The cytotoxicity was assessed by MTT assay and expressed as the percentage of optical density (%OD) in reference to the OD of amrubicin-untreated cells. Data are presented as the mean ± standard deviation of three separate experiments.

by STI571 in SCF-treated H209 cells may be attributable to functional deficiency of the Rb protein in the H209 cells.

These observations may account for the failure of the STI571-monotherapy against SCLC. Even though c-kit is

expressed, STI571 may not be effective in cases where growth-promoting signals other than SCF/c-kit are dominant, as in H69 cells. Assuming SCF/c-kit mediated activation of Akt and Erk, STI571 as a single agent may not be growth-inhibiting for SCLC cells, as observed in H209 cells since Rb protein is deficient in a majority of SCLC (18).

Although STI571 did not exert cell growth inhibitory activity in SCF-treated SCLC cells, this agent inhibited SCF-induced Akt and Erk activation in H209 cells. It has been reported that inhibition of the PI3K/Akt pathway sensitizes SCLC cells to chemotherapeutic agents (19). Accordingly, even if STI571 is ineffective as a single agent in the treatment of SCLC, there is still a possibility that this agent may be effective when combined with a conventional chemotherapeutic agent. Based on this hypothesis, we explored the effect of SCF and STI571 on the cytotoxicity induced by several chemotherapeutic agents.

In H69 cells, the sensitivity to CDDP, VP-16, CPT-11 or AMR did not change regardless of the treatment with SCF. However, in H209 cells, treatment with SCF interfered with the cytotoxicity of AMR, but not of any other agents, suggesting that SCF-induced drug resistance may be drug-specific. Since SCF did not influence the Akt or Erk activity of H69 cells in our experimental condition, it is natural that the sensitivity to the chemotherapeutic agents was not affected by the treatment with SCF in H69 cells.

To clarify the mechanism by which SCF confers AMR resistance to H209 cells, the effects of an Akt or Erk inhibitor on AMR sensitivity were evaluated in SCF-treated cells. An Akt inhibitor, LY294002, restored SCF-induced AMR resistance to the basal level in H209 cells. Interestingly, although SCF did not affect AMR sensitivity in H69 cells, the treatment with LY294002 enhanced the cell growth inhibitory activity of AMR. In addition, an Erk inhibitor, PD98059, did not have any effect on SCF-induced AMR resistance in H209 cells or AMR sensitivity in H69 cells. These observations suggest that Akt activity, but not Erk activity, is strongly related with AMR sensitivity and the suppression of Akt activity sensitizes SCLC cells to AMR.

Similar to LY294002, STI571 inhibited SCF-induced Akt activation and restored SCF-induced AMR resistance in H209 cells. Therefore, recovery of AMR sensitivity to the basal level by STI571 is attributable to the inhibition of SCF-induced Akt activation.

The observation of the present study suggests that if the SCF/c-kit-mediated autocrine signal activates Akt in SCLC *in vivo*, STI571 may enhance the anti-tumor activity of AMR through Akt inhibition, even though STI571 as a single agent is ineffective in terms of tumor growth inhibition. Furthermore, regardless of the active SCF/c-kit-mediated autocrine loop, we propose that Akt inhibition is a promising strategy to sensitize SCLC to AMR.

In terms of combination therapy using STI571, there are two reports in which STI571 was combined with CDDP and CPT-11, or carboplatin and CPT-11 in the treatment of SCLC (14,20). Neither report showed improved results by the addition of STI571 compared to the results expected with chemotherapy alone. In the present study, STI571 did not affect the cytotoxicity of either CDDP or CPT-11 in both SCF-treated H209 and H69 cells, in accordance with these reports.

However, our observation suggests the possibility that AMR is optimal as a chemotherapeutic agent combined with STI571 in the treatment of SCLC. AMR, a totally synthetic 9-amino-anthracycline, demonstrated excellent anti-tumor activity as a single-agent or combination therapy for ED-SCLC (6,21). Therefore, given that SCF/c-kit-mediated autocrine signal activates Akt in SCLC *in vivo*, the combination of STI571 and AMR may be promising in the treatment of SCLC.

Phosphorylated Akt was detected in 68% of the tumor specimens from SCLC patients (22), suggesting a high incidence of activated PI3K/Akt pathways in SCLC cells. Multiple neuropeptides and polypeptides other than SCF are proposed to function in SCLC (23). Therefore, it is still unknown whether SCF/c-kit is a dominant autocrine loop in SCLC and which growth factor should be addressed as a molecular target. Recently, we reported that the inhibition of c-Src leads to Akt suppression and the combination of c-Src-inhibiting agents with AMR exert synergistic activity in c-kit-negative N417 SCLC cells (24), consistent with the present study. Although an Akt-suppressing agent in SCLC still remains to be established, our observation that Akt suppression enhanced AMR cytotoxicity may lead to a novel strategy in treating SCLC.

In conclusion, if SCF/c-kit contributes to Akt activation *in vivo*, our study proposes a novel combination treatment of STI571 and AMR against SCLC. The enhancement of AMR cytotoxicity by STI571 may be attributed to the suppression of SCF-induced Akt activation by STI571 treatments. Although Akt-suppressing agents remain to be developed, we believe that our study provides evidence supporting the future use of combination chemotherapy using Akt inhibitors and AMR against SCLC.

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