

STI571 sensitizes nasopharyngeal carcinoma cells to cisplatin: Sustained activation of ERK with improved growth inhibition

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Abstract. Previously, we demonstrated that c-kit and stem cell factors (SCF) commonly co-expressed in primary and metastatic nasopharyngeal carcinomas (NPC), and in HONE-1 NPC cells with tyrosine autophosphorylation of c-kit. These findings suggest that the SCF/c-kit signaling may contribute to pathogenesis of NPC. Therefore, the efficacy of STI571 treatment alone and when combined with cisplatin on HONE-1 cells were evaluated. STI571 induced growth inhibition at the IC₅₀ concentration (14.9 μ M). When the concentration was at or higher than 30 μ M, the induction of cell apoptosis was observed. The effects of STI571 were shown to be mediated by the sustained activation of ERK but did not involve the inhibition of c-kit signal activity. When the STI571 (5 μ M) and cisplatin (5 μ g/ml) treatments were combined, there were further inductions of ERK activation resulting in obviously enhanced growth inhibition and induction of cell apoptosis. In a xenograft model, STI571 (50 mg/kg/day) showed only a limited ability to inhibit HONE-1 cell growth, but when combined with cisplatin (3 mg/kg/twice a week) treatment, there was a significant improvement in growth inhibition compared with STI571

or cisplatin treatment alone. Our results provide experimental support for the advanced NPC therapeutic trials using the combined STI571 and cisplatin treatment.

Introduction

Protein tyrosine kinases (PTKs) play an important role in the cellular communication network and act as master function switches. Their regulation of intracellular signals leads to a variety of cellular responses, such as cell development, proliferation, survival, apoptosis and differentiation (1). The dysregulation of PTK signaling results in malfunctions of cellular signaling transductions and this converts into various effects such as oncogenesis and proliferative disorders (2). Therefore, these PTKs have become targets in cancer therapy with the aim of blocking cancer cell growth, metastasis and angiogenesis together with the triggering of cancer cell apoptosis (3). The tyrosine kinase inhibitor, STI571 (GleevecTM, imatinib mesylate), was originally designed to interact with the ATP-binding site of PTKs and to specifically inhibit the tyrosine kinase of abl or chimeric bcr-abl. This is mediated by binding with the inactive conformation of the abl kinase resulting in the inhibition of phosphorylation, which is required for signal transduction. Such signals are essential for cell proliferation and the inhibition of cell apoptosis (4). Through this inhibition mechanism, STI571 has been shown to have remarkable clinical efficacy with minimal side effects as a first-line treatment for chronic myelogenous leukemia (5), and there have been clinical trials of the drug's use to treat bcr-abl positive acute lymphoblastic leukemia (6).

STI571 also has been shown to inhibit two closely related type III receptor tyrosine kinases, the platelet-derived growth factor receptor (PDGFR) and a stem cell factor (SCF) receptor (c-kit) (7). Clinical or pre-clinical studies have shown that STI571 has the potential to treat PDGFR-expressing tumors, such as dermatofibrosarcoma protuberans (8), glioblastoma (9), prostate (10), and PDGFR α rearrangement chronic myelogenous leukemia (11). STI571 has also been shown to inhibit bleomycin-induced pulmonary fibrosis (12) and pig serum-induced hepatic fibrosis in rats (13), which involved the inhibition of PDGFR. In addition, STI571 therapy seems to

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Abbreviations: PTK, protein tyrosine kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal regulate kinase; MEK, MAPK/ERK kinase; PARP, poly(ADP-ribose) polymerase; EBNA1, Epstein-Barr nuclear antigen 1; LMP1, latent membrane protein 1; NPC, nasopharyngeal carcinoma

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have also reduced bone marrow fibrosis in patients with chronic myelogenous leukemia (14). These results suggest that STI571 may provide an effective novel strategy for anti-fibrosis therapy that is mediated through the inhibition of PDGFR. By inhibiting c-kit, STI571 showed selective efficacy in patients with gastrointestinal stromal tumors (GIST) associated with mutational constitutional activation of c-kit, which resulted in malignant transformation (15). The inhibition of mutational c-kit activation by STI571 proved that a specific inhibitor was able to counteract the effects of this genetic mutation, which was responsible for the neoplastic transformation (16). STI571 has been applied not only to the treatment of metastatic GIST but also has been tested as an adjuvant therapy after the resection of primary GIST (17). Other pre-clinical and clinical studies of various cancers with mutational or non-mutational c-kit activation resulting in cell proliferation and antiapoptotic effects, such as mastocytosis (18), small cell carcinoma of lungs (19,20), Ewing's sarcoma cells (21,22), and colorectal carcinoma (23) have been affected by STI571. STI571 has also been demonstrated to have an inhibitory effect on the phosphorylation of wild-type c-kit in Ewing's sarcoma cells at a dose (20 μ M) higher than that required for inhibiting mutant c-kit (21). Moreover, pre-clinical studies have demonstrated that STI571 may be used in combination with conventional chemotherapeutic drugs, such as drugs interfering with DNA metabolism and this had a synergistic effect on primary chronic myelogenous leukemia cells, thus improving chronic myelogenous leukemia treatment (24). Although the combined therapeutic mechanisms have not been well clarified, similar combination therapeutic trials have been suggested and evaluated in other types of cancer (25).

Previously, we demonstrated co-expression of the c-kit receptor and its ligand SCF in primary and metastatic nasopharyngeal carcinomas (NPC) as well as in NPC HONE-1 cells. Autoactivation of c-kit was also detected in HONE-1 cells and the expression level of c-kit and its phosphorylation status were not obviously influenced by the co-expression of Epstein-Barr nuclear antigen 1 (EBNA1) and the latent membrane protein 1 (LMP1) of Epstein-Barr virus (EBV) (26). These findings suggest that the SCF/c-kit activation loop may contribute in the NPC pathogenesis. Therefore, a new treatment option was hypothesized for patients with NPC. The purpose of this study was to evaluate the therapeutic efficacy and molecular mechanism of STI571 alone and in combination with cisplatin on HONE-1 cells *in vitro* as well as in xenograft tumor system involving NPC HONE-1 cells.

Materials and methods

Reagents and antibodies. STI571 (imatinib mesylate) was provided by Novartis Pharma AG (Basel, Switzerland). Rabbit polyclonal anti-c-kit (C19), anti-phosphorylated ERK antibodies, mouse monoclonal anti-phosphorylated tyrosine (PY20) and anti-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, Los Angeles, CA, USA). Rabbit polyclonal anti-phosphorylated Akt antibody and MEK inhibitor (U0126) were from Cell Signaling Technology (Beverly, MA, USA). MEK inhibitor (PD98059) was from Calbiochem (Merck, Darmstadt, Germany). Mouse monoclonal

anti-ERK, -PARP and -LMP1 antibodies were from NeoMarkers (CA, USA). Cisplatin was from Sigma (St. Louis, MO, USA).

Cell culture, plasmid DNA and transfection. The EBV-negative NPC cell line (HONE-1), which is derived from NPC tissue, was cultured in DMEM medium containing 10% fetal calf serum (FCS), and incubated at 37°C in an incubator with 5% CO₂ and water saturation. The construction of the pCP4 control plasmid and pCP4/EBNA1 containing EBNA1-gene has been described previously (26). Construction of the LMP1-containing plasmid, pCDNA3/LMP1 has also been described (27). The pCP4/EBNA1 and pCDNA3/ LMP1 plasmids were used to cotransfect HONE-1 cells to evaluate whether the co-expression of EBNA1 and LMP1 in HONE-1 NPC cells would alter sensitivity to STI571 using an MTT assay. DNA cotransfection of the HONE-1 cells was performed using effectene transfection reagent according to the manufacturer's instructions (Qiagen, Hilden, Germany).

Assessment of growth inhibition and cell death. The MTT assay was used to evaluate the growth inhibition and cytotoxicity caused by treatment with STI571 or/and cisplatin as previously described (28). The cell death induced by STI571 or/and cisplatin and the protective efficiency of the MEK inhibitors against the cell death induced by STI571 or/and cisplatin was determined using trypan blue staining.

Western immunoblotting and immunoprecipitation. Western immunoblotting was used to evaluate the expression of EBNA1 and LMP1 in the pCP4/EBNA1 and pCDNA3/LMP1 cotransfected HONE-1 cells as well as the expression and cleavage of PARP, and the expressions of actin, ERK and Akt in HONE-1 cells, in the manner previously described (27). Immunoprecipitation plus Western immunoblotting was used to evaluate c-kit expression and its autophosphorylation in HONE-1 cells. These were performed as described previously (26).

Therapeutic trials in xenograft nude mice. HONE-1 cells (2x10⁶) were injected s.c. into 8-week-old male nude mice (*nu/nu*, Balb/c). When the tumor volume reached about 50-100 mm³, the animals were subgrouped into four groups (n=12 each), which had the same mean of tumor volume, and were treated with STI571 (50 mg/kg) by intraperitoneal injection everyday, cisplatin (3 mg/kg) by intraperitoneal injection twice a week, a combined STI571 and cisplatin, and a vehicle buffer (PBS) control for 24 days. The tumor mass present in each mouse was monitored at 3-day intervals and calculated using the following formula: $V = 0.4 \times a \times b^2$, where V represents the volume of tumor mass, a is the largest diameter of the tumor mass, and b is the smallest diameter of the tumor mass. The animals were sacrificed on the 24th day of the treatment course, and tumor masses were excised, weighted and divided into two parts. One of them was fixed in 10% neutralized formalin for histopathological assessment and the other part was stored at -80°C for various studies. In this therapeutic trial, animal behavior and body weight were also monitored every day. All of the procedures that involved animal experimentation and their care in this study were

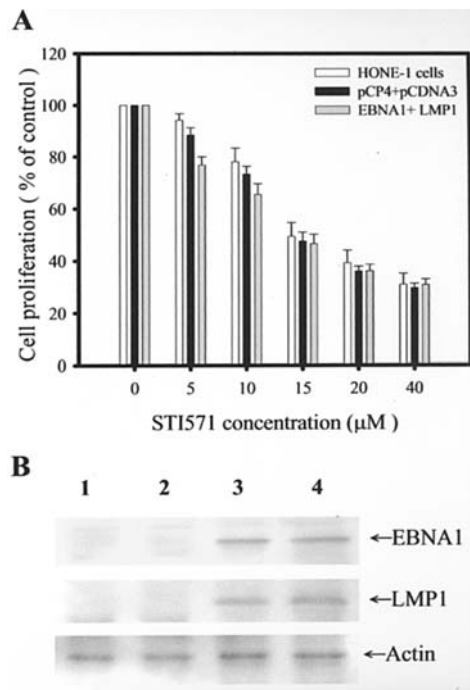


Figure 1. STI571 induces growth inhibition of HONE-1 cells and HONE-1 cells with co-expression of EBNA1 and LMP1. (A) The IC_{50} values of STI571 are for HONE-1 cells (14.9 μ M), EBNA1 and LMP1 co-expressing HONE-1 cells (14.1 μ M) and pCP4 and pCDNA3 cotransfected HONE-1 cells (14.5 μ M). These were evaluated using MTT assay after the cells were treated with STI571 for 72 h at various concentrations. The significance of differences were analyzed using Student's t-test. $P < 0.05$, the difference was considered statistically significant. pCP4+pCDNA3, HONE-1 cells cotransfected with the pCP4 and pCDNA3 plasmids as a control; EBNA1+LMP1, HONE-1 cells cotransfected by pCP4/EBNA1 and pCDNA/LMP1 with co-expression of EBNA1 and LMP1. (B) The expression of EBNA1 and LMP1 in HONE-1 cells. HONE-1 cells (column 1), pCP4 and pCDNA3 cotransfected HONE-1 cells (column 2), pCP4/EBNA1 and pCDNA/LMP1 cotransfected HONE-1 cells (column 3) and B95.8 cells (column 4) were evaluated using Western blotting. B95.8 cells were included to verify the expression level of EBNA1 and LMP1 in the HONE-1 cells. They were also probed with actin antibody for quantification.

done in accordance with an established animal protocol and approved by the Institutional Animal Care and Use Committee at the National Defense Medical Center.

Results

Growth inhibition of HONE-1 cells induced by STI571. The STI571 anti-proliferation effects in HONE-1 cells were determined by culturing HONE-1 cells in growth medium containing 10% FBS with various concentrations of STI571 (0, 5, 10, 15, 20, and 40 μ M) for 72 h, and cell proliferation was quantified using the MTT assay. HONE-1 cell proliferation was inhibited by STI571 in a dose-dependent manner (Fig. 1A). The half-maximal inhibition of cell growth was observed at 14.9 μ M of STI571 (IC_{50}). NPCs are always associated with EBV infection and commonly co-express EBNA1 and LMP1. Therefore, we also evaluated the anti-proliferation effect of STI571 on HONE-1 cells with co-expression of EBNA1 and LMP1, which is similar to the expression level in B95.8 cells (Fig. 1B). The IC_{50} of STI571 for EBNA1 and LMP1 co-expressing HONE-1 cells is 14.1 μ M, which was not significantly different from that of HONE-1

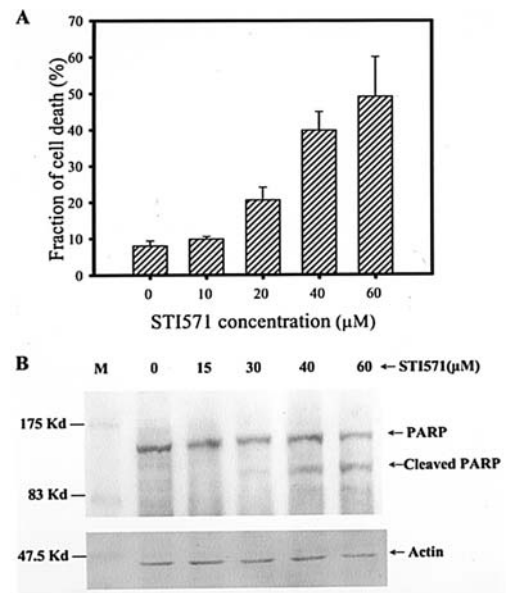


Figure 2. STI571 induced HONE-1 cell death involves the activation of caspase activity. (A) The fraction of HONE-1 cell death induced by STI571 treatment for 16 h at various concentrations was determined using manual cell counting after trypan blue stain. (B) After treatment with STI571 as indicated various concentrations for 16 h, whole cell lysates were prepared and Western immuno-blotted to evaluate the activation of caspases based on the cleavage of PARP. Actin was probed for quantification.

cells and control plasmid cotransfected HONE-1 cells (14.5 μ M) ($P = 0.423$, Fig. 1A). The IC_{50} value of STI571 for HONE-1 cells was obviously higher than that of chronic myelogenous leukaemia and GIST cells while they were similar to previous reports of the c-kit positive solid tumors, were less sensitivity to STI571, such as Ewing's sarcoma cells, lung small cell carcinoma cells and colon carcinoma cells (19-21,23). In addition, the sensitivity to STI571 of HONE-1 cells was not obviously influenced by the co-expression of EBNA1 and LMP1.

Induction of HONE-1 cell apoptosis by STI571 at higher concentration. Next, we examined STI571-induced cell death in HONE-1 cells by incubating the cells in growth medium containing 10% FBS with various concentrations of STI571 (0, 10, 20, 40, and 60 μ M) for 16 h. The cell death fraction was determined using manual cell counting after trypan blue staining of pooled non-adherent and adherent cells. As shown in Fig. 2A, there was no obvious increase in cell death that was induced by STI571 when the concentration was less than 10 μ M compared with the cells without STI571 treatment. When the concentration was at or above 20 μ M, the fraction of dead cells increased in a dose-dependent pattern with sequential characteristic morphological changes. The cells became refractile and detached from the base surface of the culture flask. To further evaluate whether STI571 induction of HONE-1 cell death was through the caspase-dependent apoptotic pathway, HONE-1 cells were treated with various concentrations of STI571 (0, 15, 30, 40, and 60 μ M) for 16 h and both attached and detached cells were harvested. The whole cell proteins were extracted and the activated caspase activity was assessed based on the cleavage of poly(ADP-ribose) polymerase (PARP), a substrate of caspase 3 and 7,

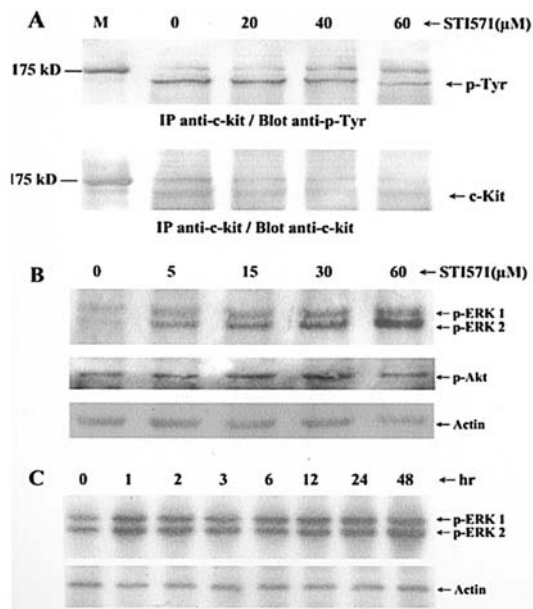


Figure 3. STI571 does not inhibit c-kit signal activity but paradoxically induces ERK activation in HONE-1 cells. (A) After treatment with STI571 as indicated at various concentrations for 16 h, whole cell lysate was immunoprecipitated with c-kit antibody and the tyrosine phosphorylation of c-kit was measured using Western immunoblotting with antiphosphotyrosine antibody (PY20) (upper panel). The same blot was stripped and reprobed with anti-c-kit antibody displaying the expression level of c-kit (lower panel). (B) After treatment with STI571 at indicated various concentrations for 16 h, whole cell lysates were prepared and Western immunoblotted to evaluate the phosphorylated activation of ERK and Akt. The blot was sequentially probed for phosphorylated ERK and Akt. Actin was probed for quantification. (C) The HONE-1 cells were treated with STI571 (5 μM) at indicated different time course. The Western immunoblotting showed STI571 is able to induce the sustained activation of ERK.

using Western immunoblotting. The cleavage of PARP induced by STI571 was observed at or higher than the concentration of 30 μM (Fig. 2B). These findings may suggest that at or below the concentration of IC₅₀ (14.9 μM) STI571 induced HONE-1 cell growth inhibition and was unlikely to induce cell apoptosis except at a higher concentration.

STI571 did not influence c-kit phosphorylation but induced sustained ERK activation in HONE-1 cells. To determine whether STI571-induced growth inhibition and apoptosis was mediated by the inhibition of c-kit activity, HONE-1 cells were treated with STI571 at various concentrations (0, 20, 40 and 60 μM) for 16 h and then the expression level and tyrosine phosphorylation status of c-kit were examined using immunoprecipitation and Western blotting probed with c-kit antibody and anti-phosphotyrosine antibody (PY20). It was demonstrated that the expression level and tyrosine phosphorylation status of c-kit in HONE-1 cells was not obviously influenced by STI571 treatment (Fig. 3A). The major downstream signaling molecules of c-kit, ERK and Akt, were also evaluated using Western immunoblotting. We found that ERK underwent phosphorylated activation by STI571 in a dose dependent manner (0, 5, 15, 30 and 60 μM), but no increased phosphorylated activation of AKT was observed at the same time (Fig. 3B). In addition, it is unknown whether the sustained phosphorylated ERK activation induced by

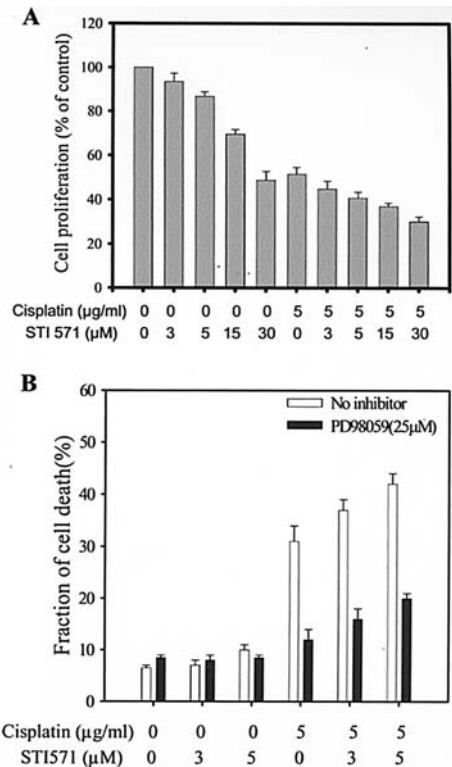


Figure 4. Cooperative cytotoxicity in HONE-1 cells was induced by combined treatments with STI571 and cisplatin. (A) An enhanced growth inhibition was induced by combined treatments with STI571 (3, 5, 15, 30 μM) and cisplatin (5 μg/ml) for 16 h when assayed using MTT assay and tested using Student's t-test. If $P < 0.05$, the difference was considered statistically significant. (B) The MEK inhibitor (PD98059) was used to verify that the cell death induced by STI571, cisplatin, or combined treatment is mediated by activation of ERK. After HONE-1 cells were treated with STI571 (3 or 5 μM), cisplatin (5 μg/ml) or combination of both for 16 h, the fraction of dead cells was determined using trypan blue exclusion.

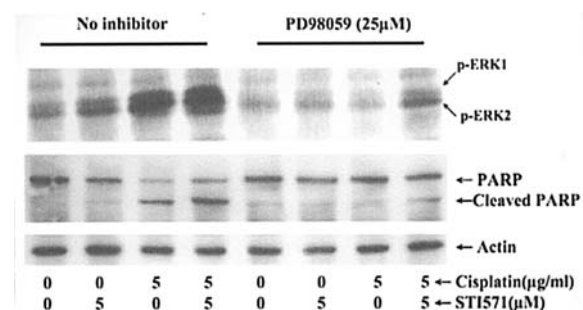


Figure 5. Cooperative effect between STI571 and cisplatin involves enhanced ERK activation. ERK phosphorylated activation was induced by STI571 (5 μM) or cisplatin (5 μg/ml) and an enhanced ERK activation was induced by a combination of cisplatin and STI571 treatment. The results were assessed using Western blot after 16-h treatment. The degree of ERK phosphorylation is positively correlated with the degree of PARP cleavage. After pretreatment with MEK inhibitor (PD98059, 25 μM) the degree of reduced ERK phosphorylation is also correlated with the degree of reduction in PARP cleavage. The blot was sequentially probed for phosphorylated ERK, PARP, and actin.

STI571 was also evaluated using Western immunoblotting. After HONE-1 cells were treated using STI571 (5 μM) for

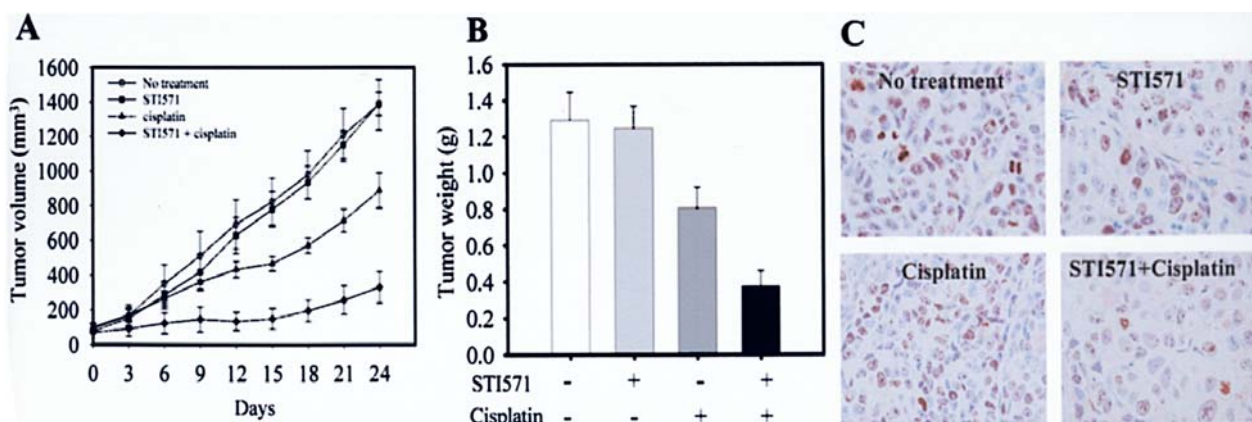


Figure 6. Therapeutic efficacy of STI571 (50 mg/kg/day), cisplatin (3 mg/kg/ twice a week), and a combination of both on HONE-1 cells. This was evaluated using a xenograft model. The therapeutic efficacy was evaluated based on the mean tumor volume tested using Student's t-test. (A), Mean tumor weight tested using Student's t-test (B), and mean proliferation index of the tumors tested using Chi-square test (C). $P < 0.05$, the difference was considered statistically significant.

different time periods (0, 1, 2, 3, 6, 12, 24, and 48 h), we demonstrated that sustained phosphorylated ERK activation was induced by STI571 at different time points (Fig. 3C). These results suggest that STI571 induced sustained ERK phosphorylated activation in HONE-1 cells. It is conceivable that the inhibition of HONE-1 cell proliferation induced by STI571 is not mediated by the inhibition of c-kit signal activity but it is possibly mediated by the sustained phosphorylated activation of ERK.

Cooperative cytotoxicity induced by STI571 and cisplatin involved the enhanced activation of ERK. Cisplatin is commonly used as an adjuvant chemotherapeutic drug for NPC. In addition, cisplatin has been shown to induce cell apoptosis mediated by sustained ERK activation (29). Therefore, we evaluated the effects of a combined treatment of STI571 and cisplatin on HONE-1 cells using the MTT assay. We showed that a combination treatment of STI571 (3, 5, 15, or 30 μ M) with cisplatin (5 μ g/ml) for 16 h resulted in a cooperative growth inhibition in HONE-1 cells was observed at the low concentration of STI571 (3 μ M) and reach statistical significance when the STI571 concentration at or higher than 5 μ M was used compared with STI571 or cisplatin treatment alone ($P < 0.01$; Fig. 4A). These results suggest that combining STI571 with cisplatin treatments on HONE-1 cells resulted in cooperative growth inhibition from a low concentration of STI571 (3 μ M).

To evaluate whether the cell death was induced by STI571, cisplatin, or combined STI571 and cisplatin treatment and to verify which was mediated by the activation of ERK, the MEK inhibitor (PDPD98059, 25 μ M) was used. After HONE-1 cells were treated with STI571 (3 or 5 μ M), cisplatin (5 μ g/ml) or combined both for 16 h, the fraction of dead cells was determined using trypan blue exclusion. The HONE-1 cell death was not obviously induced by STI571 treatment when compared with the results of the no treatment control; whereas the cell death induced by cisplatin was enhanced when combined with STI571 treatment. After pretreatment with the MEK inhibitor (PDPD98059, 25 μ M), the fraction of cell death induced by STI571 treatment was

not influenced. However, the cell death induced by cisplatin or combined with STI571 was obviously reduced by pretreatment with MEK inhibitor (Fig. 4B). These findings suggest that at a low concentration (3 or 5 μ M), STI571 will not efficiently induce HONE-1 cell death but STI571 sensitized the HONE-1 cells to cisplatin treatment and this mechanism was possibly mediated by the enhanced activation of ERK.

To clarify the cooperative mechanism between STI571 and cisplatin, we evaluated the possibility that enhanced ERK activation was induced by combined STI571 and cisplatin treatment in HONE-1 cells using Western blot analysis. After HONE cells were treated with STI571 (5 μ M), cisplatin (5 μ g/ml), or both for 16 h, we found that there was phosphorylated activation of ERK. This was not only induced by STI571 or cisplatin treatment alone, but it was also obviously enhanced by the combined STI571 and cisplatin treatment (Fig. 5). The cleavage of PARP was induced by cisplatin treatment but not induced by STI571. In addition, the increased PARP cleavage was associated with the combined STI571 and cisplatin treatment. To further verify whether the ERK activation is required for STI571, cisplatin or combined both to induce apoptotic cleavage of PARP, the specific MEK inhibitors (PD98059 and U0126) were used and evaluated using Western blot analysis. After pretreatment with the MEK inhibitor (PD98059, 25 μ M), an obviously reduced ERK phosphorylation was observed with STI571, cisplatin, or combined treatments. At the same time, the association with reduced PARP cleavage was also observed with cisplatin or combined STI571 and cisplatin treatments (Fig. 5). A similar finding was obtained after pretreatment with U0126 inhibitor (data not shown). These results showed that the MEK inhibitors inhibited apoptotic PARP cleavage mediated through the inhibition of ERK activation when treated with cisplatin alone and with a combination of STI571 and cisplatin.

These findings are compatible with the results shown in Fig. 4B and suggest that treatment with STI571 (5 μ M) induced phosphorylated activation of ERK which was unable to efficiently induce HONE-1 cell apoptosis. The treatment with cisplatin (5 μ g/ml) induced ERK phos-

phorylated activation and resulted in HONE-1 cell apoptosis. However, when we combined the treatment with STI571, which sensitized the HONE-1 cell to cisplatin, the results were an increased induction of cell apoptosis mediated by the cooperative enhanced ERK phosphorylated activation. In addition, activation of ERK was required for STI571 and cisplatin to induce HONE-1 cell growth inhibition or apoptosis.

Combined STI571 and cisplatin therapeutic efficacy in a xenograft model. The *in vitro* findings showed that STI571 induced mild HONE-1 cell growth inhibition within the therapeutic concentration ($<6 \mu\text{M}$) mediated by ERK activation and these effects sensitized the HONE-1 cells to cisplatin treatment resulting in cooperative cytotoxicity mediated by enhanced phosphorylated activation of ERK. To further evaluate the therapeutic efficacy of STI571 (50 mg/kg/day) treatment or the treatment combined with cisplatin (3 mg/kg/twice a week), an *in vivo* xenograft model system using HONE-1 cells in nude mice was used. In the xenograft model, STI571 had a limited ability to inhibit HONE-1 cell growth and there were no significant differences compared with the untreated group ($P>0.5$). There was significant inhibitory effect for cisplatin treatment compared with the untreated or STI571 treatment groups ($P<0.0001$). Importantly, there were significant enhanced therapeutic effects of the combined STI571 and cisplatin treatment compared with no treatment, treatment with STI571, or treatment with cisplatin alone ($P<0.0001$) (Fig. 6A). As shown in Fig. 6B, a similar significantly better effect of the combined STI571 and cisplatin treatment compared to no treatment, treatment with STI571 or treatment with cisplatin alone was obtained when this was assessed using tumor weight ($P<0.0001$). Quantification of the cell proliferation index of the tumor mass derived from the various treated and untreated groups using immunohistochemical staining with Ki-67 antibody (MIB1) showed that the proliferative index of the tumors derived from the combined STI571 and cisplatin treatment group (42%) was lower than that of the cisplatin treatment group (55%, $P=0.089$) and reached statistical significance when compared with that of the tumors derived from the STI571 treatment group (71%, $P=0.0001$) or the untreated group (75%, $P=0.0001$) (Fig. 6C). These results suggest that combined STI571 and cisplatin treatment has significantly enhanced therapeutic effects compared with STI571 or cisplatin treatment alone in the xenograft model. There were no significant undesired effects of this therapeutic trial on animal behavior or body weight.

Discussion

In a previous study, we demonstrated that c-kit and SCF were commonly co-expressed in primary and metastatic NPCs, and in NPC HONE-1 cells with a constitutive tyrosine phosphorylation of c-kit and it was not obviously influenced by the co-expression of EBNA1 and LMP1. These findings suggest that the SCF/c-kit activation loop possibly contribute in the pathogenesis of NPC. In the present study, we evaluated the therapeutic efficacy of STI571 or combined cisplatin treatment on HONE-1 cells. We have shown that STI571

inhibited HONE-1 cell proliferation in a dose-dependent manner and the IC_{50} is $14.9 \mu\text{M}$ at 72 h of treatment. These results are similar to the findings in other studies of c-kit-positive solid tumor cells, such as lung small cell carcinoma, Ewing sarcoma and colon carcinoma cells (19-21,23) with IC_{50} values of STI571 usually higher than that of chronic myelogenous leukemia and GIST tumor. Such tumors express SCF and c-kit, but the biological effects and significance of the SCF/c-kit loop and its involvement in cell proliferation and survival are still not well clarified. They are commonly less sensitive to STI571 treatment than those with chronic myelogenous leukemia and GIST, which may be explained by the following possibilities: limited uptake of STI571, the SCF/c-kit loop is not essential for cell survival and proliferation, a great variety of intracellular inhibition action, or inhibition of other tyrosine kinases. In addition, we have shown that the anti-proliferation effects of STI571 in HONE-1 cells were not significantly influenced by co-expression of EBNA1 and LMP1 (IC_{50} value of $14.1 \mu\text{M}$ at 72-h treatment). NPCs are always associated with EBV infection and commonly co-express EBNA1 and LMP1. It is conceivable that present results on the therapeutic efficacy and mechanisms of STI571 or combined with cisplatin treatments on HONE-1 cells may also mimic the cellular environment of NPC cells with EBV infection. STI571 induced growth inhibition or apoptosis of HONE-1 cells are mediated by the ERK activation but do not involve the inhibition of c-kit signal activity because the expression level and tyrosine phosphorylation status of c-kit in HONE-1 cells are not obviously influenced by STI571 treatment at various concentrations. Within the therapeutic concentration ($<6 \mu\text{M}$), STI571 induced sustained phosphorylated activation of ERK while it was unable to efficiently induce HONE-1 cell apoptosis. However, when we combined the treatment with cisplatin, STI571 sensitized the HONE-1 cells to cisplatin resulting in an increased induction of cell apoptosis mediated by the cooperative enhanced ERK phosphorylated activation. In addition, activation of ERK is required for STI571 and cisplatin to induce HONE-1 cell growth inhibition or apoptosis.

The phosphorylated activation of ERK by STI571 in HONE-1 cells resulting in induction of cell growth inhibition or apoptosis contrasts with the general view that ERK activation plays an important role in promoting cell cycle progression and proliferation signals (30). In general, ERK activation occurs rapidly and if transient, it enhances cell survival and proliferation (31); however, if ERK activation is delayed and sustained, it induces an apoptotic signal (32). Here, we demonstrated that STI571 induced sustained ERK activation, which led to HONE-1 cell growth inhibition and induced cell apoptosis at high concentration. Similar findings to this for sustained ERK activation were noted for apoptotic induction in a number of other studies, such as cisplatin-induced apoptosis of HeLa cells (29), OK renal epithelial cells (33), A172 glioma cells (34), and calcimycin-induced apoptosis of lens epithelial cells (35). In contrast, the inhibition of ERK activation has been reported to sensitize tumor cells to chemotherapy drug induced-apoptosis including cisplatin-treated ovarian carcinoma cells (36), adaphostin-treated leukemia cells (37) and conjugated linoleic acid-treated MDA-MB-231 breast cancer cells (38). Furthermore,

it has been demonstrated that both activation and inactivation of the ERK signal activity resulted in apoptosis mediated by the mitochondria dysfunctional pathway. Based on these reports, it is clear that the underlying mechanisms of cell apoptosis mediated by the activation or inactivation of ERK signal activity are still not well understood. The mechanisms of ERK activation associated with growth inhibition and apoptosis induction in STI571 treated HONE-1 cells also remains to be clarified by further research.

STI571 induced growth inhibition that was mediated by ERK activation but did not involve the inhibition of c-kit signal activity in HONE-1 cells. A similar finding was reported in the c-kit positive neuroectodermal tumor cell lines where STI571 inhibits cell growth does not critically involve c-kit inhibition (39). In addition, c-kit-negative small cell lung cancer (40) and multiple myeloma cells (41) have also been demonstrated to be inhibited in terms of growth ability by STI571. Based on these diverse findings, it is conceivable that c-kit tyrosine kinase is not necessarily the target molecule of STI571 in some c-kit positive and negative tumors. This can be explained by the generalized phosphorylation inhibition by STI571, which also inhibits tyrosine kinases other than that of bcr-abl, PDGFR or c-kit; these other kinases must also be capable of modulating cell survival, proliferation, differentiation and death (3). This possibility has been supported recently where globalized phosphorylation inhibition by STI571 treatment was demonstrated in K562 chronic myelogenous leukemia cells (42) and in a study of some undocumented tyrosine phosphorylation sites involving various biological pathways (43). The latter were identified using multidimensional liquid chromatography and this occurred after STI571 treatment in bcr-abl positive chronic myelogenous leukemia cells.

Cisplatin is a commonly used chemotherapeutic drug for NPC and has been shown to induce caspase-dependent apoptosis in targeted cells, which is mediated by sustained ERK activation (29). We demonstrated that cisplatin induced HONE-1 cell growth inhibition and caspase-dependent apoptosis mediated by ERK activation and that this was enhanced by combined STI571 treatment leading to greater growth inhibition and cytotoxicity of HONE-1 cells. The *in vitro* findings in this study showed that within the therapeutic concentration STI571 induced growth inhibition of HONE-1 cells mediated by the sustained activation of ERK and this effect sensitized the HONE-1 cells to cisplatin resulting in enhanced cytotoxicity mediated by the enhanced ERK activation. Based on the *in vitro* results, the therapeutic efficacy of STI571 treatment alone or combined with cisplatin treatment were evaluated using a xenograft HONE-1 cell model system in nude mice. To avoid therapeutic toxicity resulting from the combined STI571 and cisplatin treatment, 50 mg/kg/day of STI571 and 3 mg/kg/twice a week of cisplatin were used to carry out these therapeutic trials. It was demonstrated that STI571 alone showed only a limited inhibition effect (not statistically significant) on HONE-1 cell growth while cisplatin showed a significant inhibitory effect, both compared with the untreated control group. A combined treatment of STI571 with cisplatin resulted in a significantly better therapeutic effect compared with the control, STI571, and cisplatin alone treatment groups.

STI571 induced growth inhibition in HONE-1 cells without involving the inhibition of c-kit signal activity *in vitro* but had a negligible therapeutic efficacy in the xenograft therapeutic model. This is compatible with the study results for c-kit-positive small cell lung cancer (44). These findings suggest that STI571 is unlikely to be effective as a monotherapy for NPC, small cell lung cancer or other c-kit-positive solid tumors. However, our *in vivo* therapeutic results were compatible with the *in vitro* findings that STI571 is able to sensitize HONE-1 cells to cisplatin resulting in a significantly efficient growth inhibition. A combination of STI571 with conventional chemotherapeutic drugs has also been studied in other c-kit positive solid tumors, such as when Ewing's sarcoma cells were treated with STI571 leading to growth inhibition, sensitized cells in the presence of vincristine and doxorubicin *in vitro* (45), and with small cell lung carcinoma cells treated using a combination of STI571 and chemotherapeutic drugs (etoposide + ifosfamide or topotecan) in a xenograft model, which resulted in enhanced growth inhibition but where the inhibition efficiency was uncorrelated with the c-kit expression level (40). In addition, the pre-clinical and clinical therapeutic trials of a combination of STI571 and various chemotherapeutic drugs and novel molecular drugs have also been reported for tumors and cancer cells other than where there was an association with c-kit. Examples include chronic myeloid leukemia cells (24,25,46,47), acute lymphoblastic leukemia cells with Philadelphia chromosome (48), gastric carcinoma (49), prostate carcinoma (50), glioblastoma (51), squamous cell carcinoma (52) and chronic lymphocytic leukemia (53). However, only some of these studies demonstrated therapeutic effects that were mediated by modulation of the known molecular targets of STI571, such as abl kinase activity, PDGFR and c-kit. It is conceivable that the detected cooperative therapeutic effects resulting from the combined treatments with STI571 and other chemotherapeutic drugs or novel molecular agents were mediated by inhibition of other tyrosine kinases or by undiscovered action mechanisms.

STI571 was administered using intraperitoneal injection at a dose of 50 mg/kg/day in our xenograft therapeutic model and this resulted in the highest plasma and intratumoral concentration of STI571 at less than 6.5 μ M and 3.8 μ M, respectively (44); this concentration resulted in only limited growth inhibition (not statistically significant) of HONE-1 cells. However, when combined with cisplatin treatment (3 mg/kg/twice a week), the results were significant cooperative growth inhibition compared with the control and treatment with cisplatin alone or STI571 alone. Thus, the cooperative interactions between STI571 and cisplatin would seem to play an important part in the induction of better therapeutic effects on HONE-1 cell xenograft tumors. Although the *in vivo* results were compatible with the *in vitro* findings, the low concentration level of STI571 *in vivo* was unable to fully explain the therapeutic effects found for STI571, which induced activation of ERK activation in the tumor cells and enhancement after combination with cisplatin treatment. Therefore, additional biological action mechanisms of STI571 need to be proposed that facilitate the therapeutic efficacy and result in the enhancement when STI571 is combined with cisplatin treatment *in vivo*. These may include:

a) general inhibition of phosphorylation of tyrosine kinase that sensitizes cells to cisplatin-induced cell death (42,43) or other down-regulation of cisplatin resistant mechanisms (54); b) inhibition of platelet-derived growth factor (PDGF) receptors that will result in reduced interstitial hypertension, which, in turn, leads to increased transcapillary transport of STI571 into the tumor (55); c) inhibition of tumor angiogenesis by STI571 mediated through the blockage of angiogenesis induced by PDGF, VEGF (vascular endothelial growth factor), bFGF (basic fibroblast growth factor) and SCF/c-kit activity *in vivo* (7,56); d) increased INF- γ production by NK cells that is induced by STI571 and results in enhanced anti-tumor effects (57); and e) inhibition of cytochrome p450 activity by STI571, which may decrease the elimination of any chemotherapeutic agent (58).

In conclusion, we demonstrated that STI571 induced growth inhibition of HONE-1 cells that were mediated by sustained ERK activation but did not involve the inhibition of c-kit signal activity. The cooperative cytotoxicity of STI571 and cisplatin combined treatment resulted from the mediation of enhanced ERK activation and this was found within the therapeutic concentration (<6 μ M) of STI571 *in vitro*. Moreover, significantly better therapeutic effects on growth inhibition of HONE-1 cell xenograft tumors were obtained using the combined treatment of STI571 and cisplatin. The results highlight that clinical trials for advanced NPC using a combined treatment of STI571 and cisplatin or other chemotherapeutic drugs may be warranted based on our experimental findings.

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