STI571 (Glivec) induces cell death in the gastrointestinal stromal tumor cell line, GIST-T1, via endoplasmic reticulum stress response

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Received December 20, 2005; Accepted February 6, 2006

Abstract. STI571 is a specific inhibitor of tyrosine kinases, such as BCR-ABL, platelet-derived growth factor receptor, and c-KIT, and has recently been approved for the treatment of chronic myeloid leukemia and gastrointestinal stromal tumors (GISTs). This study demonstrated that STI571 induces cell death in the gastrointestinal stromal tumor cell line, GIST-T1. In these cells, STI571 induced pro-caspase-12 or pro-caspase-7 cleavage and it affected caspase-3 activity and induced the endoplasmic reticulum (ER)-resident chaperone, glucose-regulated protein 78. The STI571-induced cell death was blocked by the protein synthesis inhibitor, cycloheximide. Together, these results suggest that STI571 induces cell death in GIST-T1 cells, at least in part, via the ER stress response.

Introduction

Stress conditions in cells up-regulate chaperone proteins and repress protein synthesis (1,2). In the endoplasmic reticulum (ER), stress conditions induce a cellular defense mechanism referred to as the unfolded protein response (URP) (1,2). One characteristic of this response is the induction of the ER-resident chaperones, including the glucose-regulated proteins (GRPs) (1,2). GRP78 increases protein folding in the ER lumen and is the best characterized of these proteins. It is up-regulated during the ER stress response, and when ER stress signals are unable to rescue cells, cell death pathways are activated. ER stress is induced by exposure to tunicamycin and brefeldin A. Tunicamycin is a nucleoside antibiotic that inhibits *N*-linked glycosilation of target asparagine residues in the luminal domain proteins, whereas brefeldin A blocks the translocation of proteins from the ER to the Golgi aparatus by causing disassembly of the Golgi complex (3). The mechanism of cell death by ER stress remains incompletely elucidated; however, caspase-12 and -7 have been implicated in addition to the crucial effector caspase-3 (1-3).

Gastrointestinal stromal tumors (GISTs) are thought to be mesenchymal in origin, being derived from cells of the digestive tract. GISTs are characterized by the expression of both c-KIT and CD34 on the plasma membrane. Recent studies linked a mutation in *c-kit* to the process of oncogenic activation (4,5). The *c-kit* proto-oncogene encodes for a tyrosine kinase receptor. Binding of the ligand, stem cell factor (SCF), activates the tyrosine kinase function of c-KIT, allowing it to transduce signals to the nucleus by phosphorylation of tyrosine residues in recruited signaling proteins. It is thought that deregulation of the tyrosine kinase activity is one of the principal mechanisms involved in abnormal cell growth and survival of malignant cells.

Recently, protein kinase inhibitors that disrupt these autonomous signaling loops have been developed for clinical use (6-12). STI571 was designated as an inhibitor of BCR/ABL, platelet-derived growth factor receptor (PDGFR), and c-KIT (13), and has been used successfully in unresectable or metastatic GIST patients with constitutive activation of c-KIT (10,11).

In this study, we used the GIST-T1 cell line, which was established from a patient with metastatic GIST (14). To our knowledge, GIST-T1 is one of only two GIST cell lines that exist. GIST-T1 cells have a heterogenic *c-kit* mutation in exon 11 and c-KIT in GIST-T1 cells is constitutively activated (14,15). We treated GIST-T1 cells with STI571 to assess the effects on cell growth and cell death, and the mechanisms by which such effects might be mediated.

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Key words: gastrointestinal stromal tumor, c-KIT, STI571, GIST-T1, endoplasmic reticulum stress response

Materials and methods

Reagents. Dulbecco's modified Eagle's medium (DMEM) and antibiotics (penicillin, streptomycin) were purchased from Invitrogen Corporation (New York, USA). Anti-caspase-12 antibody, anti-caspase-7, and anti-GRP78 antibody were purchased from Santa Cruz Biotechnology, Inc. (CA, USA). Tunicamycin and brefeldin A were purchased from Wako (Osaka, Japan). The CPP32/caspase-3 colorimetric protease assay kit was purchased from Medical Biological Laboratories Co. Ltd. (Nagoya, Japan). Cycloheximide was purchased from Sigma (St. Louis, USA). STI571 (Glivec capsule, Novartis, Basel, Switzerland) was diluted in water to 5 μg/μl and stored at -20˚C. Caspase-3 inhibitor (ApoAlert DEVD-CHO) was purchased from Clontech Laboratories (CA, USA).

Cells and cell culture. GIST-T1 cells were cultured in DMEM supplemented with penicillin, streptomycin, and 8% fetal bovine serum (FBS) maintained in a 95% air, 5% CO_2 atmosphere at 37˚C in a humidified incubator.

MTT assay to determine cell viability. In a 96-well plate, $1x10^6$ cells/100 μl of cell suspension were used to seed each well. After 24 h, reagents (STI571, brefeldin A, tunicamycin) were added in each well and cells were incubated for 24 h. After a 24 h incubation with/without reagents, 10 μl of a 2.5 mg/ml solution in PBS of the MTT (3,-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide; Sigma-Aldrich, Tokyo, Japan) was added in each well and incubated for 2 h at 37˚C. The resulting violet formazan precipitate was solubilized by the addition of 100 μl of a 50% N,N,-Dimethyl formamide/10% SDS solution, and incubated for 4 h at room temperature. The plate was then analyzed on a plate reader at 570 nm to measure sample absorbance.

Flow cytometry. Cells ($1x10^6$) were seeded on a 6-cm plate and incubated for 24 h. Reagents were then added: STI571 (1 μg/ml), tunicamycin (2 μg/ml), or brefeldin A (1 μM). To inhibit protein synthesis, cells were pre-treated with cyclohexmide (100 μg/ml) for 1 h before the addition of reagents. After 24 h, both adherent and non-adherent cells were trypsinized and washed twice with ice-cold PBS and the cells were centrifuged at 500 x g for 5 min. Cells were treated with Annexin V-FITC Reagent (BioVision, Palo Alto, CA, USA) according to the manufacturer's protocol. Flow cytometry (FACscan, Becton Dickinson) was then used to determine the percentage of apoptotic cells with high FITC signal among ~10000 events (using CellQuest software, Becton Dickinson).

Western blotting. Cells ($4x10^6$) were seeded on a 10-cm dish for 24 h prior to the addition of reagents, STI571, brefeldin A, or tunicamycin in medium for a 24 h incubation. Cells were washed with PBS and lysed in RIPA buffer (Upstate Biotechnology Inc., NY, USA) containing 20 mM sodium pyrophosphate, 20 mM NaF, 1 mM orthovanadate, 2 mM pyrophosphate, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Cell lysates containing comparable amounts of proteins, estimated by a Bradford assay (Bio-Rad, Munchen, Germany) were separated by SDS-PAGE and subjected to Western blotting.

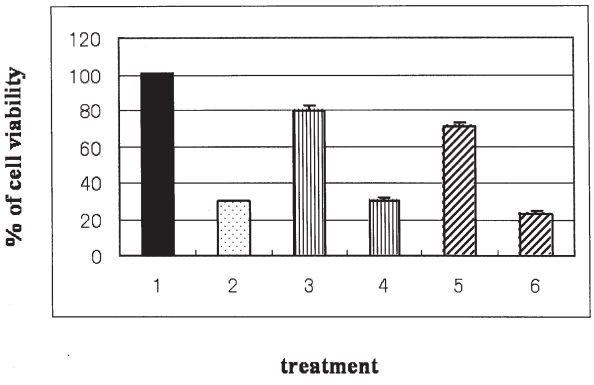

Figure 1. Cell viability was determined by MTT assay. Treatments: 1, non-treated; 2, STI571 (1 μg/ml); 3, tunicamycin (0.1 μg/ml); 4, tunicamycin (2 μg/ml); 5, brefeldin A (0.1 μM); 6, brefeldin A (1 μM). The survival percentages are shown as the mean ± standard deviation of triplicate experiments.

Determination of caspase-3 activity. Cells prepared as described for Western blotting were washed with PBS and lysed in the buffer supplied with the CPP32/caspase-3 colorimetric protease assay kit. The assay was carried out according to the manufacturer's protocol. The absorbance of each sample was measured at 405 nm on a plate reader.

Results

Assessment of cell viability by MTT assay. The cell viability of the GIST-T1 cells decreased in a dose-dependent manner following treatment with STI571 (15). At the concentration of 1 μg/ml, STI571 reduced the viability to 30.1±0.5%. Tunicamycin (2 μg/ml) and brefeldin A (1 μM) also affected the cell viability of the GIST-T1 cells (30.2±1.5% and 23.5±1.0%, respectively) (Fig. 1).

Determination of cell death. Both annexin V- and propidium iodide (PI)-positive cells represented necrotic or delayed-phase apoptotic cells (upper right quadrant). On the other hand, Annexin V-positive and PI-negative cells represented the early-phase apoptotic cells (lower right quadrant). STI571 (Fig. 2B), brefeldin A (Fig. 2C), and tunicamycin (Fig. 2D) induced mainly necrotic or delayed phase apoptotic cells following a 24 h incubation period (15.8, 27.3, 20.6%, respectively). Brefeldin A and tunicamycin are known to cause ER stress and induce cell death via the ER stress response (1,16). In a previous study, Tinhofer *et al* reported that cycloheximide prevented cell death due to ER stress response (17). Cycloheximide is an inhibitor of protein synthesis, and it prevented excessive protein accumulation in the ER. Excessive protein accumulation in the ER caused cell death via ER stress response. Furthermore, we tested whether cycloheximide prevented cell death in the GIST-T1 caused by STI571 (Fig. 2F), tunicamycin (Fig. 2H), or brefeldin A (Fig. 2G). Cycloheximide did not induce cell death in the GIST-T1 cells, and it blocked the cell death caused by STI571, brefeldin A or tunicamycin treatment to levels of 5.5, 8.4, or 6.8%, respectively.

GRP78 expression and cleavage of pro-caspase-12 or pro-caspase-7. Cells under stress conditions accumulate unfolded

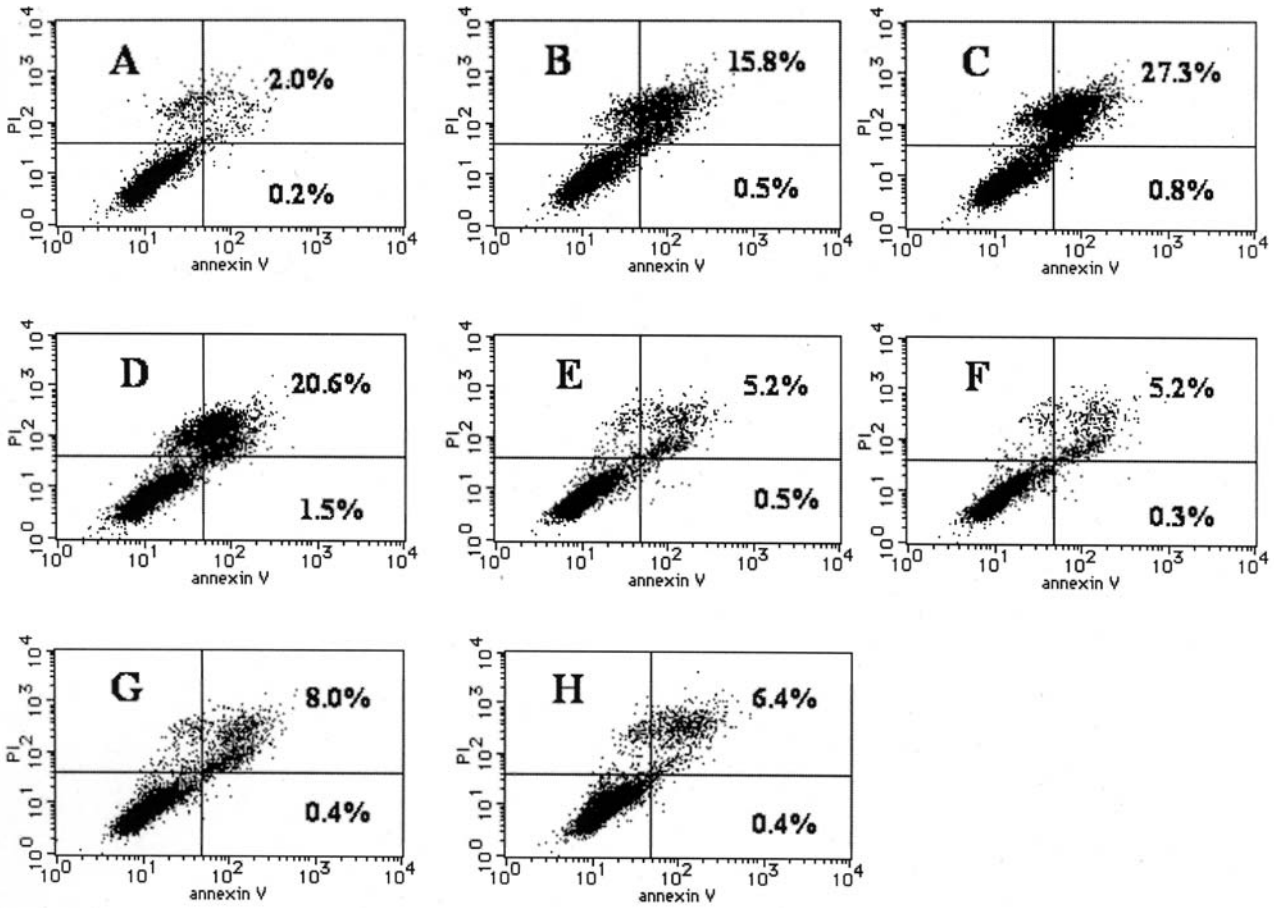

Figure 2. Cell death assay. Cells were treated with STI571, tunicamycin, or brefeldin A, with/without pre-treatment with 100 μg/ml of cycloheximide (CHX). A, non-treated; B, STI571 (1 μg/ml); C, brefeldin A (1 μM); D, tunicamycin (2 μg/ml); E, CHX; F, CHX + STI571 (1 μg/ml); G, CHX + brefeldin A (1 μM); H, CHX + tunicamycin (2 μg/ml).

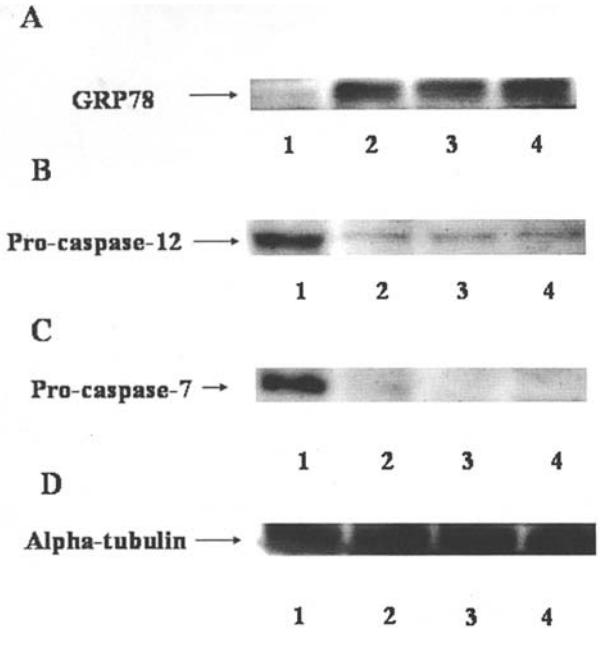

Figure 3. Determination of GRP78, pro-caspase-12, or pro-caspase-7 was determined by Western blot analysis. Cells were treated with STI571, brefeldin A, or tunicamycin for 24 h. Lane 1, non-treated; 2, STI571 (1 μg/ml); 3, brefeldin A (1 μM); 4, tunicamycin (5 μg/ml). (A) Expression of GRP78, (B) cleavage of pro-caspase-12, (C) cleavage of pro-caspase-7, (D) expression of alpha-tubulin for control. Three independent experiments were done and similar results were obtained.

protein response-induced ER-resident chaperones such as GRP78 (1,2). GRP78 acts to protect the cells from stress; however, cells subjected to excessive stress undergo cell death via caspase-12, -7 and -3 activation (1,2,16,18). In this study, Western blot analysis revealed that STI571, brefeldin A, and tunicamycin up-regulated the expression of GRP78 (Fig. 3A).

Furthermore, we investigated the expression and cleavage of caspase-12 or -7. In this study, we used anti-mouse caspase-12 antibody for determination of caspase-12 in the GIST-T1 cells. In a previous study, Hetz *et al* reported that anti-mouse caspase-12 antibodies could be detectable for human caspase-12 (18). Xie *et al* reported that pro-caspase-12 was observed as a 60-kDa band in the human liver-derived Huh7 cell line (19). Pro-caspase-12 in the Huh7 cells was cleaved by reagent which induced ER stress (19). In our study, pro-caspase-12 in the GIST-T1 was detected as an ~60-kDa band and it was cleaved by treatment with STI571, brefeldin A, or tunicamycin (Fig. 3B). Pro-caspase-7 was also cleaved by STI571, brefeldin A, or tunicamycin (Fig. 3C).

Caspase-3 activity. In the cell death mechanism caused by ER stress response, caspase-3 was activated followed by caspase-12 and -7 activation (16,18). Caspase-3 is well known as a key molecule which is involved in the cell death pathway. Therefore, we assessed whether caspase-3 was activated

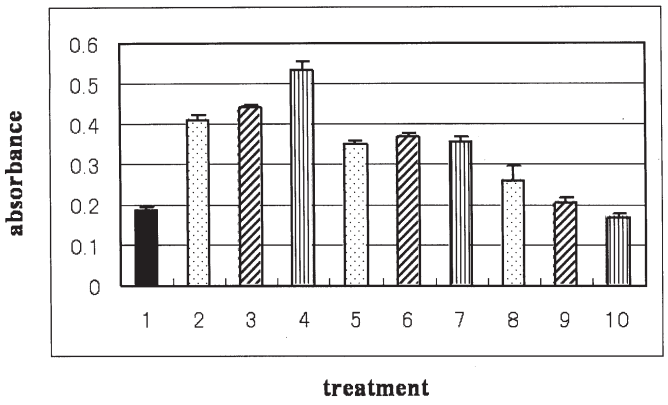

Figure 4. Caspase-3 activity. Caspase-3 activity was measured by using the CPP32/caspase-3 colometric protease assay kit, and the results were represented as absorbance. Treatment: 1, non-treated; 2, STI571 (1 μg/ml); 3, brefeldin A (1 μM); 4, tunicamycin (5 μg/ml); 5, cycloheximide (CHX) + STI571 (1 μg/ml); 6, CHX + brefeldin A (1 μM); 7, CHX + tunicamycin (5 μg/ml); 8, caspase-3 inhibitor (10 μM) + STI571 (1 μg/ml); 9, caspase-3 inhibitor (10 μM) + brefeldin A (1 μM); 10, caspase-3 inhibitor (10 μM) + tunicamycin (5 μg/ml). The results are shown as the mean ± standard deviation of triplicate experiments.

followed by ER stress response in the GIST-T1 cells. Caspase-3 activity was measured by using the CPP32/caspase-3 colometric protease assay kit, and the results were represented as absorbance. STI571 led to caspase-3 activation (absorbance; 0.411±0.011). Tunicamycin, or brefeldin A also led to caspase-3 activation (absorbance; 0.531±0.023, 0.441±0.005, respectively). Cycloheximide attenuated caspase-3 activation by STI571, brefeldin A, or tunicamycin (absorbance; 0.348±0.01, 0.355±0.01, 0.367±0.011, respectively). Caspase-3 inhibitor also attenuated caspase-3 activation by STI571, brefeldin A, or tunicamycin (absorbance; 0.259±0.035, 0.204±0.013, 0.168±0.011, respectively). Absorbance of non-treated cells was 0.185±0.008. STI571 as well as brefeldin A or tunicamycin activated caspase-3 and cycloheximide attenuated the activation of caspae-3 induced by STI571, brefeldin A, or tunicamycin (Fig. 4).

Discussion

In our previous study, we demonstrated that STI571 inhibited the activation of c-KIT and reduced the cell viability in the gastrointestinal stromal tumor cell line, GIST-T1 (15). STI571 not only reduced cell proliferation but also induced cell death in GIST-T1 cells. The cell death mechanism which is induced by STI571 is not clear. It is very important to clarify the cell death mechanism which is induced by STI571 in GIST cells.

Recent studies revealed that endoplasmic reticulum stress response was one of the cell death mechanisms. Cells under stress conditions accumulate unfolded protein response-induced ER-resident chaperones such as GRP78 (1,2). GRP78 acts to protect the cells from stress; however, cells subjected to excessive stress undergo cell death via caspase-12, -7, and -3 activation (16,18,19). Brefeldin A and tunicamycin are known to cause ER stress and induce cell death via the ER stress response (3). In the GIST-T1 cells studied herein, STI571, brefeldin A and tunicamycin all markedly reduced cell viability and induced cell death, and blocking protein synthesis in these cells using cycloheximide prevented cell death induced by STI571, brefeldin A, or tunicamycin. Cycloheximide prevented excessive protein accumulation in the ER. These results suggested that STI571 induced cell death via ER stress response as well as tunicamycin or brefeldin A. The mechanism of cell death by ER stress remains incompletely elucidated; however, caspase-12 and -7 have been implicated in addition to the crucial effector caspase-3 (1-3). Next, we observed whether the expression of GRP78 and activation of caspase-12, -7, or -3 by treatment with STI571, brefeldin A, or tunicamycin. STI571 induce the expression of GRP78 and activated caspase-12, -7, or -3. Cycloheximide or caspase-3 inhibitor attenuated the activation of caspase-3 by treatment with STI571, brefeldin A, or tunicamycin. These results suggested that caspase-3 was activated followed by caspase-7, caspase-12 with STI571, as well as brefeldin A or tunicamycin treatment. In our study, cisplatin which is well known as an anti-cancer reagent induced cell death of GIST-T1 at 10 μg/ml concentration, but cycloheximide could not prevent cell death induced by cisplatin (data not shown). Therefore, the STI571-induced cell death pathway was different from the cisplatin-induced one. Our previous study revealed that STI571 inhibited the activation of c-KIT in the GIST-T1 (15). Furthermore, we tested whether stem cell factor (SCF), c-KIT ligand, could rescue the cell death in the GIST-T1 cells. SCF could not rescue the cell death of GIST-T1 cells induced by STI571 (data not shown). STI571 completely inhibited the activation of c-KIT in the GIST-T1 cells even at stimulation with high concentration of SCF (100 ng/ml) (data not shown). STI571 did not induce cell death of another cancer cell line, such as DLD-1 (colon adenocarcinoma), MKN-45 (gastric cancer), SK-N-MC (neuroblastoma) (data not shown). These results indicated that activation of c-KIT played a crucial role in the survival signal in the GIST-T1 cells.

Taken together, STI571 inhibited activation of c-KIT and induced cell death of the GIST-T1 cells, at least in part, via ER stress response. Further studies are required to clarify the functions of molecules that modulate the cell death pathway, including mitochondrial cell death pathway induced by STI571 treatment.

Acknowledgements

We thank Ms. Motoko Miyata and Ms. Yuka Takezaki for their technical assistance.

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