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SHORT REPORTS

STI571 inactivation of the gastrointestinal stromal tumor c-KIT oncoprotein: biological and clinical implications

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Mutations in the *c-KIT* receptor occur somatically in many sporadic Gastrointestinal Stromal Tumors (GIST), and similar mutations have been identified at the germline level in kindreds with multiple GISTs. These mutations activate the tyrosine kinase activity of c-KIT and induce constitutive signaling. To investigate the function of activated c-KIT in GIST, we established a human GIST cell line, GIST882, which expresses an activating KIT mutation (K642E) in the first part of the cytoplasmic split tyrosine kinase domain. Notably, the K642E substitution is encoded by a homozygous exon 13 missense mutation, and, therefore, GIST882 cells do not express native KIT. GIST882 c-KIT protein is constitutively tyrosine phosphorylated, but tyrosine phosphorylation was rapidly and completely abolished after incubating the cells with the selective tyrosine kinase inhibitor STI571. Furthermore, GIST882 cells evidenced decreased proliferation and the onset of apoptotic cell death after prolonged incubation with STI571. Similar results were obtained after administering STI571 to a primary GIST cell culture that expressed a *c-KIT* exon 11 juxtamembrane mutation (K558NP). These cell-culture-based studies support an important role for c-KIT signaling in GIST and suggest therapeutic potential for STI571 in patients afflicted by this chemoresistant tumor. *Oncogene* (2001) **20,** 5054–5058.

Keywords: Gastrointestional Stromal Tumors (GIST); c-KIT; STI571

GISTs are the most common mesenchymal tumors of the stomach and proximal small intestine, with an estimated yearly incidence of at least 2000–5000 cases in the US (Miettinen *et al*., 1999; Nishida and Hirota, 2000; CDM Fletcher, personal communication). GISTs are typically discovered either incidentally during endoscopic, radiologic, or surgical procedures; or are diagnosed in the evaluation of patients presenting with an abdominal mass, abdominal pain, or upper gastrointestinal bleeding. Surgical resection with curative intent is the approach to patients presenting with localized GIST; however, many patients with localized GIST ultimately relapse, and unresectable and metastatic GIST has a poor prognosis because systemic chemotherapy and local radiotherapy are ineffective (Ueyama *et al*., 1992; Edmonson *et al*., 1999; DeMatteo *et al*., 2000).

Until recently, GISTs were classified most often as smooth muscle tumors. However, contemporary histopathological criteria, particularly expression of the *c-KIT* receptor tyrosine kinase (CD117), enable true non-myogenic GISTs to be discriminated from other gastrointestinal mesenchymal tumors (De Saint Aubain Somerhausen and Fletcher, 1998; Sarlomo-Rikala *et al*., 1998; Chan, 1999; Miettinen *et al*., 1999; Rubin *et al*., 2000; Nishida and Hirota, 2000). Sequencing of the *c-KIT* gene has revealed activating mutations in many GISTs, and GISTs lacking *c-KIT* mutations may have a better prognosis (Hirota *et al*., 1998; Ernst *et al*., 1998; Moskaluk *et al*., 1999; Lasota *et al*., 1999; Taniguchi *et al*., 1999). A key role for c-KIT in GIST pathogenesis is supported by the finding that some kindreds with an autosomal dominant pattern of multiple primary GISTs have germline *c-KIT* activating mutations (Nishida *et al*., 1998). The mechanisms of GIST c-KIT activation appear to be varied, as suggested by the finding of oncogenic mutations in the extracellular, juxtamembrane, and kinase domains (Lux *et al*., 2000; Lasota *et al*., 2000).

To examine the relevance of c-KIT signaling in the pathobiology of GIST, we established a GIST cell line, GIST882, from a patient with metastatic GIST. This GIST cell line is the first to be reported, and has been in continuous culture for two years. Both the primary GIST and the GIST882 cell line expressed a *c-KIT* allele with an exon 13 missense mutation, resulting in a single amino acid substitution, K642E, in the proximal part of the split tyrosine kinase domain. The K642E c-KIT oncoprotein, in GIST882

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cell lysates, had constitutive kinase activity (data not shown). In addition, wild-type *c-KIT* transcripts were not detected in either the primary tumor or in GIST882, and GIST882 did not proliferate in response to supplemental stem cell factor (SCF), indicating that this cell line represented a unique opportunity to examine mutant c-KIT signaling independent of the wild-type c-KIT signaling pathway (data not shown). Cytogenetic characterization revealed monosomy of chromosomes 14 and 22 in the GIST882 cell line and primary tumor (data not shown).

C-KIT enzymatic activity can be inhibited by the tyrosine kinase inhibitor STI571, a 2-phenylaminopyrimidine which selectively inhibits proto-oncogenic and oncogenic forms of the *ABL*, *PDGFR*, and *c-KIT* tyrosine kinases (Druker *et al*., 1996; Buchdunger *et al*., 1996; Carroll *et al*., 1997; Krystal *et al*., 2000; Wang *et al*., 2000; Heinrich *et al*., 2000). To examine the effects of STI571 on GIST882, cells were cultured with different concentrations of drug for 90 min, and immunoprecipitates were then examined for loss of c-KIT tyrosine phosphorylation. Preliminary experiments demonstrated that a concentration of 1 μM STI571 resulted in complete inhibition of c-KIT tyrosine phosphorylation over this incubation period, and thus this dose was chosen for subsequent studies. Upon incubation of GIST882 with 1 μM STI571, examination of whole cell lysates revealed decreased tyrosine phosphorylation of a 145 kD protein by 1 h of incubation, and this 145 kD tyrosine phosphoprotein co-migrated with c-KIT (Figure 1a). C-KIT immunoprecipitates from these same lysates demonstrated a parallel diminution of 145 kD c-KIT tyrosine phosphorylation (Figure 1b), whereas c-KIT protein levels were unchanged (Figure 1c). C-KIT immunodepletion from control cell lysates revealed that the 145 kD tyrosine phosphorylated species could be substantially depleted (data not shown).

STI571 anti-proliferative effects were determined by culturing GIST882 and four comparison sarcoma cell lines in various concentrations of STI571, and then quantitating cell proliferation by manual cell counting. Over a 5 day period, GIST882 proliferation was consistently inhibited by STI571 concentrations $\geqslant 0.1\ \mu$M (Figure 2). In contrast, cell proliferation was not inhibited by even 10 μM STI571 in any of the four comparison sarcoma cell lines, including the malignant peripheral nerve sheath tumor cell line ST88-014 (Figure 2, Reynolds *et al*., 1992), the fibrosarcoma cell line HT-1080 (not shown, Rasheed *et al*., 1974), and immortal cell lines established from malignant peripheral nerve sheath tumors and mesotheliomas (data not shown). Although ST88-014 has been reported to express c-KIT and respond to exogeneous SCF (Badache *et al*., 1998), we did not supplement the cultures with SCF and therefore could not determine whether STI571 inhibits the KIT/SCF pathway in MPNST 88-014.

GIST882 cells, during prolonged incubations with STI571, became refractile and detached from the

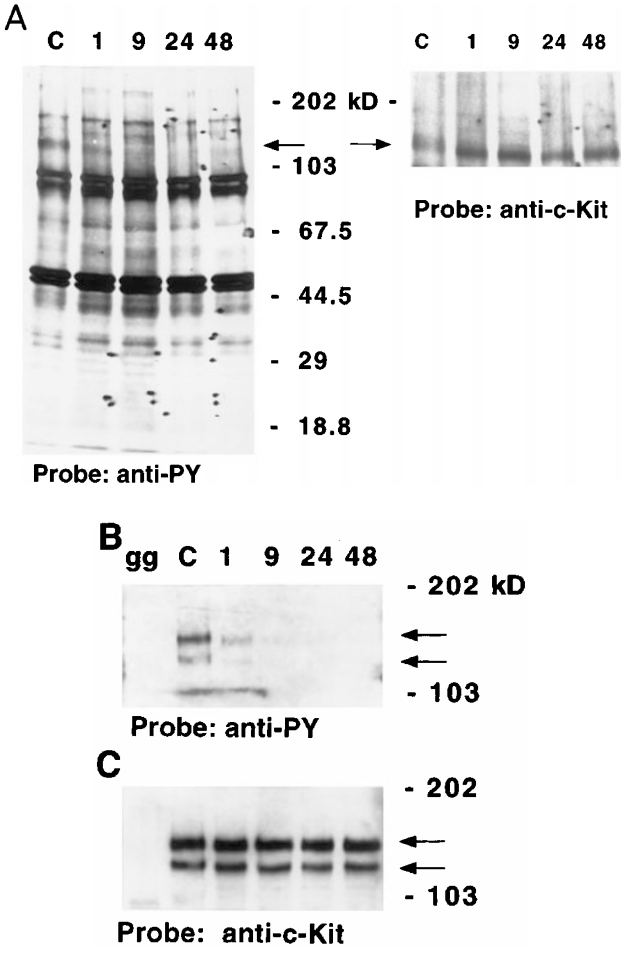

Figure 1 Selective inhibition of c-Kit tyrosine phosphorylation in the GIST cell line GIST882 by treatment with STI571. GIST882 were maintained in 10% FCS/DMEM/0.5% mitotracker plus (Becton Dickinson)/1% BPE, and all treatments were performed in 10% FCS/DMEM. Treatment involved the addition of a 1% volume of PBS or STI571 dissolved in PBS to the media. Cell lysis buffer, immunoprecipitation and Western blotting methods were as described (Tuveson *et al*., 1993). (**a**) Western blotting of whole cell lysates reveals a tyrosine phosphorylated 145 kD species which is present in GIST882 incubated with control media (media with 1% PBS) for 48 h (first lane, labeled 'C'). This 145 kD tyrosine phosphoprotein is lost selectively after incubation with media containing 1 μM STI571 dissolved in PBS for 1–48 h (lanes labeled 1, 9, 24, 48). Tyrosine phosphorylated proteins were detected with 4G10-biotin (UBI) followed by Streptavidin–HRP (Pierce) and enhanced chemiluminescence (ECL, Amersham). The blot was stripped and reprobed with anti-c-KIT antibody (DAKO, C19), demonstrating comigration of the 145 kD form of c-KIT with this tyrosine phosphorylated protein. The 125 kD form of c-KIT is weakly tyrosine phosphorylated, and not obvious in the analysis of whole cell lysates. (**b**) Antiphosphotyrosine Western blot of c-KIT immunoprecipitates (Santa Cruz, sc-168) prepared from the lysates in (**a**) demonstrate reduced tyrosine phosphorylation of 145 and 125 kD c-KIT forms. A control immunoprecipitation with non-immune rabbit globulin (gg) from PBS treated cells is also shown. (**c**) The blot in (**b**) was stripped and reprobed with a different c-KIT antibody (DAKO, C19) showing equivalent amounts of KIT in each immunoprecipitate

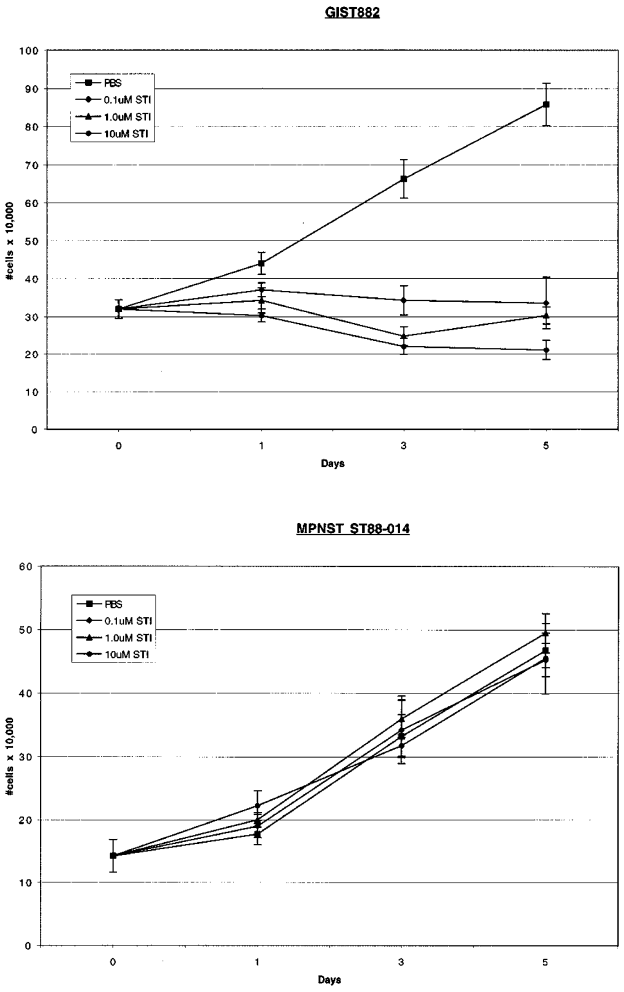

Figure 2 STI571 inhibits proliferation of GIST882 but not MPNST line ST88-014. Cells were cultured in flat bottom 24-well plates in 10% FCS/DMEM, and treated in quadruplicate with a range of STI571 for the indicated times. Cells were trypsinized, and viable cells which excluded Trypan blue were quantitated on a hemocytometer, with the results given with standard deviations

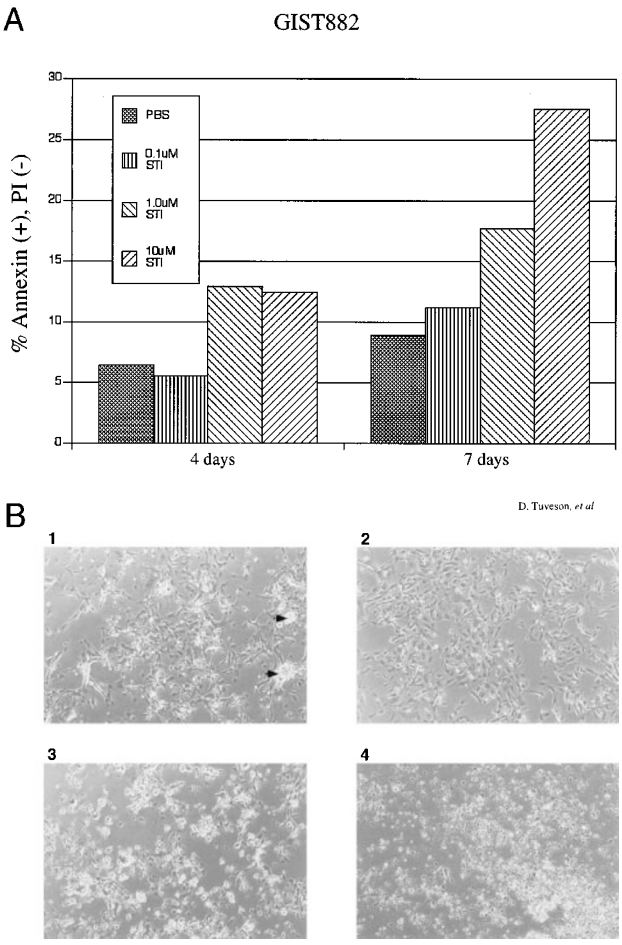

Figure 3 Induction of apoptosis and decreased focus formation in GIST882 after prolonged culture with STI571. (**a**) Cells were incubated with various concentrations of STI571 for 4 and 7 days, and early apoptotic cells which excluded propidium Iodide were quantitated by Annexin V staining and flow cytometry (Becton Dickinson). The experiment was repeated twice, with similar results. (**b**) Light microscopic analysis of GIST882 treated for 7 days with PBS (1), or STI571: 0.1 μM (2), 1.0 μM (3), and 10 μM (4). Foci are denoted in (1) with arrowheads

surface of the tissue culture flasks. However, these alterations were never observed after incubating the non-GIST sarcoma cell lines with STI571. A possible role of apoptotic cell death, in the STI571 treated GIST882 cells, was then evaluated by performing Annexin V staining on pooled non-adherent and adherent GIST882 cells and analysing by flow cytometry (Koopman *et al*., 1994). As shown in Figure 3a, the fraction of early apoptotic cells was increased 2–3-fold after STI571 incubation for 4 and 7 days, in a dose-dependent manner. In addition, we assayed for focus formation as a characteristic of transformed cells. The 7 day cultures revealed cell foci in the absence of STI571 (Figure 3b, panel 1), no foci with 0.1 μM STI571 (panel 2), and refractile and detached cells with 1 μM (panel 3) and 10 μM STI571 (panel 4). Upon quantitation, focus formation was consistently reduced by more than 20-fold in GIST882 incubated with $\geqslant$0.1 μM STI571 for 7 days.

Similar experiments were performed on a primary GIST culture established immediately after surgical resection (Figure 4). This tumor, GIST780, harbored a common exon 11 juxtamembrane mutation (K558NP). Two GIST780 cultures at identical semiconfluent density were exposed to PBS (control) or 1 μM STI571 for 48 h, and visual inspection revealed an obvious decrease in cell monolayer density in the STI571 treated culture compared with the control culture (data not shown). The GIST780 cultures did not survive continuous passaging; therefore, to quantitate the inhibition of GIST780 proliferation by STI571, cloning rings were used in triplicate to isolate representative samples from the STI571 and control treated cultures. Manual cell counting revealed 70% fewer GIST cells in the

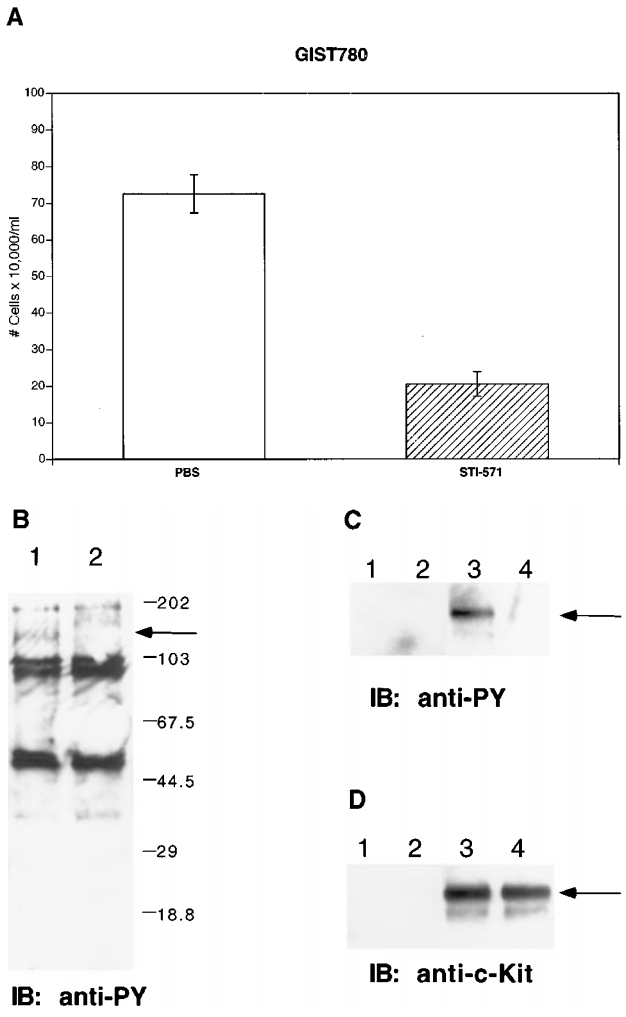

Figure 4 Inhibition of cell growth and c-KIT tyrosine phosphorylation in a primary GIST cell culture (GIST780) treated with 1 μM STI571 for 48 h. (**a**) Decreased GIST780 cell numbers after exposure to STI571. Cloning rings were used to isolate three samples from PBS and 1 μM STI571 treated cultures, and cell counts were determined. The cells were more than 95% viable by Trypan blue exclusion. The mean of the data are presented, with standard deviations. (**b**) 4G10 immunoblotting of GIST780 cell lysates after 48 h incubation with PBS (lane 1) or 1 μM STI571 (lane 2). (**c**) 4G10 immunoblotting of anti-KIT (lanes 3, 4) and non-immune rabbit IgG (lanes 1, 2) immunoprecipitates after 48 h incubation with PBS (lanes 1, 3) or 1 μM STI571 (lanes 2, 4). (**d**) Same Western blot as in (**c**), stripped and reprobed for KIT

STI571 treated cell culture compared to the control culture (Figure 4a). Interestingly, abundant foci were seen in cell monolayers of the control culture, with 58 foci counted in four low-power fields (4× magnification). In contrast, there was nearly complete inhibition of focus formation in cell monolayers of the STI571 culture, with only three foci counted in four low-power fields. In addition, cell lysates were prepared for analysis of c-KIT tyrosine phosphorylation. Immunoblotting analyses demonstrated selective loss of a 145 kD tyrosine phosphorylated protein species in STI571 treated GIST780 cells (Figure 4b), with parallel loss of tyrosine phosphorylated c-KIT (Figure 4c,d).

Two GIST cell cultures that expressed different oncogenic alleles of *c-KIT* were used to demonstrate that inhibition of constitutive c-KIT signaling with STI571 correlated with decreased proliferation, inhibition of focus formation, and the onset of apoptotic cell death. Our evidence suggests that the inhibitory effects of STI571 on GIST cells is likely due to the enzymatic inactivation of c-KIT, and not other targets. First, c-KIT is the only protein with demonstrable loss of tyrosine phosphorylation in whole cell lysates (Figures 1 and 4). Furthermore, other known targets of STI571 were either undetectable (PDGFR-α and PDGFR-β) or detected at very low levels in the inactive, unphosphorylated form (ABL) in untreated GIST882 cells (data not shown). Importantly, STI571 did not inhibit non-GIST comparison sarcoma cell cultures.

We have shown that GIST, a chemoresistant tumor, may in some cases be dependent upon constitutive signaling through oncogenic alleles of *c-KIT*. This observation extends previous work demonstrating the inhibition of c-KIT/SCF dependent cell responses and signal transduction pathways by STI571 (Krystal *et al*., 2000; Wang *et al*., 2000; Heinrich *et al*., 2000), and the inhibition of a mast cell leukemia line that expressed oncogenic c-KIT (Heinrich *et al*., 2000). Whether GISTs that express wild-type c-KIT will be inhibited by STI571 is currently unknown. Importantly, *in vivo* efficacy and safety of STI571 have already been established for patients with chronic myeloid leukemia, where STI571 serves to inhibit BCR–ABL oncogenic activity (Druker *et al*., 1999). These findings highlight a possible therapeutic benefit of STI571 in patients with locally advanced or metastatic GIST.

Note added in proof

A recently published single patient case report demonstrated a response to STI571 in GIST (Joensuu *et al*., 2001).

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