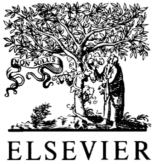

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Tyrosine kinase inhibitor STI-571/Gleevec down-regulates the β-catenin signaling activity

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Abstract

β-Catenin is a critical transducer of the Wnt signal pathway and plays an important role in many developmental and cellular processes. Deregulation of β-catenin signaling has been observed in a broad range of human tumors. In this report, we investigated whether tyrosine kinase inhibitor STI-571 could inhibit the β-catenin signaling activity and hence suppress cell proliferation. Our results demonstrated that STI-571 effectively inhibited the constitutive activity of β-catenin signaling in human colon cancer cells as well as the Wnt1-induced activation of β-catenin signaling in HOS, HTB-94, and HEK 293 cells. Furthermore, STI-571 was shown to effectively suppress the proliferation of human colon cancer cells. Finally, we demonstrated that the Wnt1-mediated activation of a GAL4-β-catenin heterologous transcription system was effectively inhibited by STI-571. Thus, our findings suggest that tyrosine phosphorylation may play an important role in regulating β-catenin signaling activity, and inhibition of this signaling pathway by STI-571 may be further explored as an important target for alternative/adjuvant treatments for a broader range of human cancer.

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Keywords: Cancer; β-Catenin; Gleevec; STI-571; Tyrosine kinase inhibitor; Wnt signal

1. Introduction

Recent studies suggest that deregulation of β-catenin signaling may play an important role in tumorigenesis [1–3]. Originally identified as a cytoplasmic protein that interacts with cell adhesion molecules, such as E-cadherin, β-catenin is an essential signal transducer of the Wnt/Wingless pathway [4,5]. Wnt ligands initiate their signaling by binding to the *frizzled* receptors, as well as the recently identified co-receptors LRP5 and LRP6, leading to phosphorylation of the *disheveled* protein.

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It then associates with Axin and the adenomatous polyposis coli (APC) tumor suppressor, preventing GSK3β from phosphorylating β-catenin. Unphosphorylated β-catenin is stabilized by escaping recognition by ubiquitin/proteosome complex, and eventually translocates to the nucleus where it engages transcription factors LEF/TCF-4 to activate the expression of downstream targets, such as c-Myc and cyclin D1 [3,6–11]. The involvement of β-catenin signaling in tumorigenesis was first established in colorectal cancer, where the β-catenin protein is stabilized by inactivating mutations of the APC tumor suppressor gene or oncogenic mutations of the β-catenin gene in the vast majority of colon cancers [1]. Recently β-catenin mutations have been detected in a variety of human tumors [12,13]. Oncogenic forms of β-catenin have been shown to induce tumor formation in transgenic animals [14,15], whereas mutations in β-catenin gene have been frequently detected in tumors induced by either carcinogens or activated oncogenes [16,17]. These collective genetic data strongly suggest that deregulation of β-catenin signaling may be involved in the development of a broad range of human malignancies.

Protein kinases play an important role in regulating cell proliferation [18,19]. Aberrant activation of some kinases has been associated with neoplastic transformation and/or tumorigenesis [19]. Targeting these kinases may represent an important therapeutic alternative for human cancer [20–22]. Recently, one such kinase inhibitor, STI-571, has gained widespread attention. STI-571 (a.k.a., Gleevec, Glivec, CGP57148, or imatinib mesylate), a small selective inhibitor of the Bcr-Abl, c-kit, and PDGF receptor tyrosine kinases, has been approved for the treatment of chronic myeloid leukemia (CML) and gastrointestinal stromal tumors (GISTs) [23–27]. Promising anti-tumor effects of STI-571 have also been reported in dermatofibrosarcoma protuberans, many of which harbor a COL1A1-PDGFB fusion protein [28,29]. As discussed above, many types of human cancer frequently exhibit a significant nuclear and/or cytoplasmic accumulation of β-catenin protein, a hallmark of deregulated β-catenin activity [3,30]. With the exception of colorectal cancer, genetic alterations of β-catenin are only detected in a small fraction of non-colon tumors, and the upstream regulators of the β-catenin signaling pathway are rarely mutated. Therefore, the molecular mechanisms underlying β-catenin deregulation remains undefined in most human tumors. Several recent studies suggest that β-catenin is a tyrosine-phosphorylated protein and some growth factors (such as HGF, IGF-1 and IGF-2) may play a role in regulating β-catenin signaling activity [31–40]. In this study, we investigated whether tyrosine kinase inhibitor STI-571 exhibits any inhibitory effect on β-catenin signaling.

2. Materials and methods

2.1. Cell lines and chemicals

Cell lines HEK 293, HOS (a human osteosarcoma cell line), and HTB-94 (a human chondrosarcoma cell line) were purchased from ATCC (Manassas, VA). Human colon cancer lines, HCT116 and SW480, were kindly provided by Bert Vogelstein of Johns Hopkins Medical Institutes, Baltimore, MD. HOS cells were maintained in complete MEM Eagle supplemented with 10% fetal bovine serum (FBS, Mediatech, Herndon, VA), 100 units of penicillin, and 100 μg of streptomycin, 1× non-essential amino acids (Mediatech), 1 mM sodium pyruvate (Mediatech) at 37 °C in 5% CO_2. HEK 293 and HTB-94 were maintained in complete DMEM containing 10% FBS and 100 units of penicillin, and 100 μg of streptomycin. HCT116 and SW480 were maintained in complete McCoy's 5A supplemented with 10% FBS and 100 units of penicillin, and 100 μg of streptomycin. STI-571 was provided by Novartis Pharmaceuticals AG (Basel, Switzerland). Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

2.2. Construction of the Wnt1 adenoviral vector and preparation of Wnt1-conditioned medium

The cDNA encoding mouse Wnt1 was tagged with a HA epitope at its C-terminus, and was subcloned into the pAdTrack-CMV vector, resulting in pAdTrack-Wnt1. Recombinant adenovirus (i.e. AdWnt1) expressing Wnt1 was generated as previously described [41]. To prepare Wnt1-conditioned medium (Wnt1-CM), the purified AdWnt1 was used to infect

exponentially growing HCT116 cells in 75 cm^2 cell culture flasks (MOI = 20). Adenovirus-containing medium was removed and washed off at 6 h after infection, and replaced with 15 ml of serum-free DMEM for each 75 cm^2 cell culture flask. Wnt1-CM was collected at 24 h and after passing through 0.2-μm filters, was kept at 4 °C.

2.3. Establishment of 293-GEM stable lines as a functional reporter system of the β-catenin signaling activity

To construct a GAL4-β-Catenin (GBC) chimeric transcription factor, the DNA-binding domain (i.e. amino acids 1–147) of the yeast transcription factor GAL4 was fused in-frame with human β-catenin [42], resulting pCMV-GBC. A reporter vector, pGRE-GFP, which expressed green fluorescent protein (GFP) under the control of a GAL4-responsive promoter, was generously provided by Bert Vogelstein of Johns Hopkins Oncology Center [43]. In order to establish a 293-GEM stable line, exponentially growing HEK 293 cells were co-transfected with 1.0 μg of pGRE-GFP plasmid DNA and 0.2 μg of pCMV-GBC plasmid per 25 cm^2 tissue culture flask by using LipofectAMINE according to the manufacturer's instructions (Life Technologies, Gaithersburg, MD). At 24 h after transfection, cells were trypsinized and replated into 96-well cell culture plates at multiple dilutions. Stable clones were selected in the presence of G418 at a final concentration of 0.3 mg/ml for 2 weeks. Clones derived from single cells were grown up for further characterization [42].

2.4. Transfection and luciferase assay

Exponentially growing cells were seeded in 25 cm^2 cell culture flasks, and transfected with 2 μg per flask of β-catenin/TCF4-responsive luciferase reporter, pTOP-Luc [44] or a control reporter, pGL3-Control (Promega, Madison, WI) using LipofectAmine (Life Technologies, Gaithersburg, MD). At 16 h after transfection, cells were replated to 48-well plates. At 12 h after replating, cells were treated with STI-571 only (for HCT116 and SW480), or STI-571 in the presence of Wnt1-CM (for HEK 293), or infection with AdWnt1 (for HOS and HTB-94). At 24–48 h after treatment, cells were lysed and cell lysates were collected for luciferase assays using Promega's Luciferase Assay Kit. Each assay condition was performed in triplicate.

2.5. Cell proliferation assay

Exponentially growing HCT116 and SW480 cells were seeded at subconfluence in 48-well cell culture plates, and treated with STI-571 at a concentration range of 0–30 μM. At 24, 48, 72, and 96 h after treatment, cells were collected by trypsinization. Viable cells were counted in the presence of Trypan Blue (Mediatech). Each assay condition was carried out in triplicate. The average of viable cells was calculated from the cell counting from the triplicate in each assay condition.

3. Results

3.1. β-Catenin/TCF4-mediated transcription activity is inhibited by STI-571

To test the possible effect of STI-571 on β-catenin signaling activity, we first introduced a TCF4-responsive reporter into two human colorectal cancer lines, HCT116 and SW480. Both cell lines contain a high constitutive activity of β-catenin signaling because the HCT116 line harbors an oncogenic mutation of the β-catenin gene, whereas SW480 contains an inactivating mutation of the APC gene [1, 44]. It has been shown that TCF4-responsive reporter pTOP-Luc was highly active in these cell lines. As illustrated in Fig. 1, in the presence of STI-571 the TCF4 reporter activity was significantly inhibited. Specifically, the TCF4 reporter activity in HCT116 cells decreased to 55, 34, 24, and 14% of that of the control in the presence of 15, 20, 25, and 30 μM of STI-571, respectively (Fig. 1A). Interestingly, in SW480 cells, 15 μM STI-571 did not exert any detectable inhibition on TCF4 reporter; however, the effect of STI-571 at higher doses was more pronounced in SW480 cells when compared to HCT116 (Fig. 1B). For instance, when SW480 cells were treated with 25 μM of STI-571, TCF4 reporter activity decreased to 7%, as opposed to 24% in HCT116 cells treated with the same concentration of STI-571. This phenomenon was reproducibly

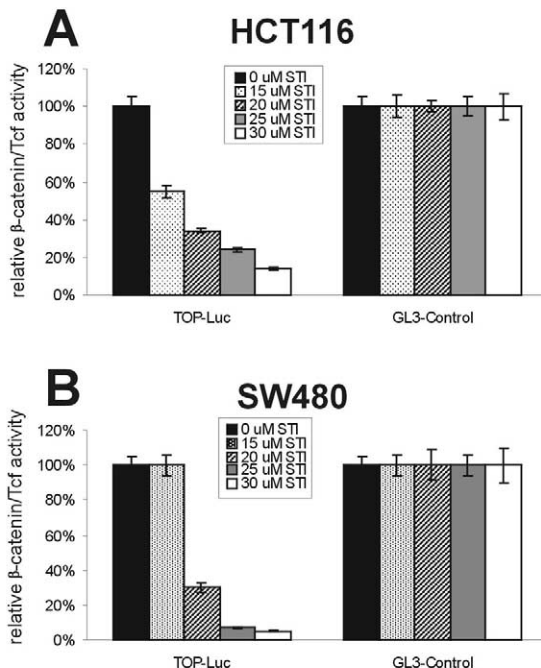

Fig. 1. STI-571 inhibits β-catenin/TCF4-mediated transcription activity in human colon cancer cell lines. Subconfluent HCT116 (A) and SW480 (B) were transfected with the TCF4 reporter pTOP-Luc or the positive control reporter pGL3-Control. The transfected cells were replated in 48-well culture plates and treated with STI-571 at the indicated concentrations. At 24 h after treatment, cells were lysed for luciferase activity assays. Each assay condition was done in triplicate.

observed in three independent batches of experiments. Conversely, using the same concentrations of STI-571, we did not observe any significant effect on pFOP-Luc, a mutant TCF4-responsive reporter in these cell lines (data not shown). Furthermore, the STI-571-mediated inhibition was seemingly specific, as the activity of pGL3-Control, a control reporter that was constitutively active under the control of SV40 promoter, was not affected under the same conditions. These results suggest that STI-571 can effectively inhibit the constitutive activity of β-catenin signaling in representative human colon cancer lines.

3.2. *Wnt1-induced β-catenin signaling activity is inhibited by STI-571*

We next studied whether STI-571 could inhibit Wnt1-induced β-catenin signaling activity in other human cells. Using a recombinant adenoviral vector expressing mouse Wnt1, we were previously able to reconstitute Wnt1-mediated activation of β-catenin signaling in the human osteosarcoma line, HOS, and the human chondrosarcoma line, HTB-94 [42]. In HOS cells, Wnt1-induced β-catenin activity decreased to 57 and 30% in the presence of 20 and 25 μM of STI-571, respectively (Fig. 2A). Interestingly, HTB-94 cells were seemingly more sensitive to STI-571 inhibition. The β-catenin/TCF4 activity was inhibited by approximately 60 and 90% when HTB-94 cells were treated with 5 and 10 μM of STI-571, respectively (Fig. 2B).

Next, we tested whether Wnt1-induced β-catenin signaling could be inhibited by STI-571 in HEK 293 cells. The above experiments involved using human tumor cells in which the deregulation of β-catenin signaling is either well established (i.e. in HCT116 and SW480) or not known (i.e. in HOS and HTB-94). We sought to test the effect of STI-571 on β-catenin signaling in the cells without any possible alterations in the Wnt/β-catenin signaling pathway. Although HEK 293 cells are transformed human embryonic kidney cells, we have demonstrated that the β-catenin signaling pathway is intact in this cell line [44,45]. Using Wnt1-conditioned medium, we were able to reconstitute the activation of β-catenin signaling in HEK 293 cells. As shown in Fig. 2C, Wnt1-CM-induced β-catenin activity was inhibited by approximately 70 and 85% in the presence of 15 and 20 μM of STI-571, respectively. These results confirm that STI-571 can effectively inhibit the Wnt/β-catenin signaling pathway.

3.3. *Proliferation of human cancer lines is inhibited by STI-571*

We tested whether STI-571-mediated inhibition of β-catenin/TCF4 activity could affect the proliferation of human cancer cells. It has been well established that the activation of β-catenin signaling is a critical early step for colon cancer development. We were interested in testing whether the growth of colon cancer cells could be significantly inhibited by STI-571. Experimentally, subconfluent HCT116 and SW480 cells were treated with different concentrations (0–30 μM) of STI-571. Viable cells were counted at 24, 48, 72, and 96 h after treatment. As shown in Fig. 3, cell proliferation was, in general,

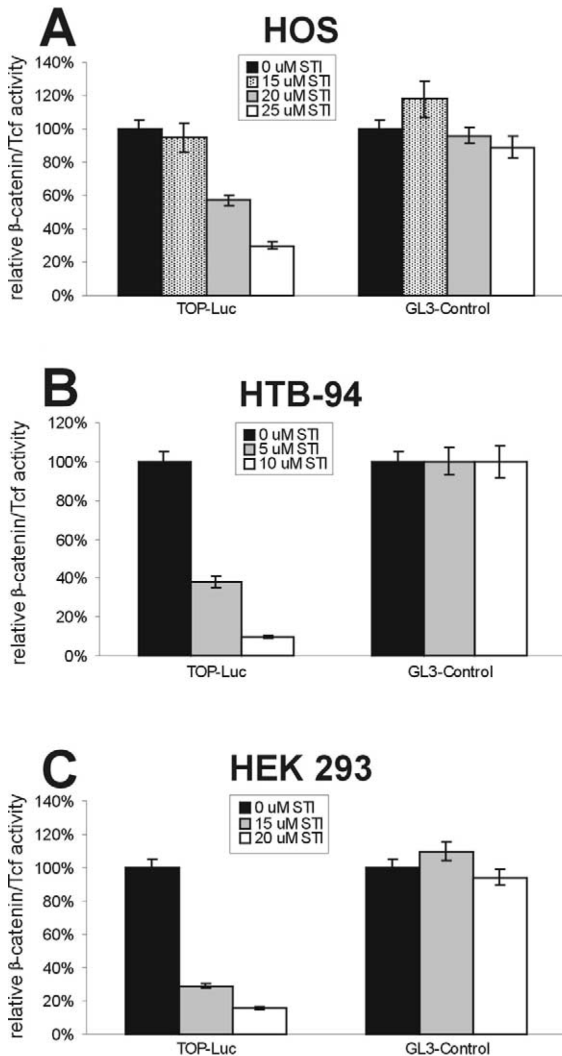

Fig. 2. STI-571 inhibits Wnt1-induced activation of β-catenin signaling. Subconfluent HOS (A), HTB-94 (B), and HEK 293 (C) were transfected with the TCF4 reporter pTOP-Luc or the positive control reporter pGL3-Control. The transfected cells were replated in 48-well culture plates and treated with STI-571 (at the indicated concentrations) with the infection of AdWnt1 (A and B), or in the presence of Wnt1-conditioned medium (C). At 48 h after treatment, cells were lysed for luciferase activity assays. Each assay condition was done in triplicate.

inhibited by STI-571 in a time- and dose-dependent manner. For instance, at 30 μM of STI-571 the number of viable HCT116 cells decreased to approximately 36, 21, and 7% of that of the controls at 48, 72, and 96 h post treatment (Fig. 3A). Similar growth inhibition by STI-571 (especially at 30 μM) was observed in SW480 cells (Fig. 3B). Interestingly, we observed a similar growth inhibitory effect of STI-571 on HTB-94 and HOS cell lines (data not shown). These results suggest that inhibition of β-catenin/TCF4 activity may be, at least in part, responsible for the STI-571-mediated growth suppression of human tumor cells.

3.4. Wnt1-mediated heterologous transactivation in 293-GEM cells is inhibited by STI-571

Finally, we sought to test whether STI-571 could effectively inhibit the Wnt1-mediated heterologous transactivation using our previously established functional assay system of β-catenin signaling [42]. This system consisted of a chimeric transcription factor (by fusing Gal4-DNA binding domain with full-length β-catenin) and a reporter (expressing GFP, driven by a

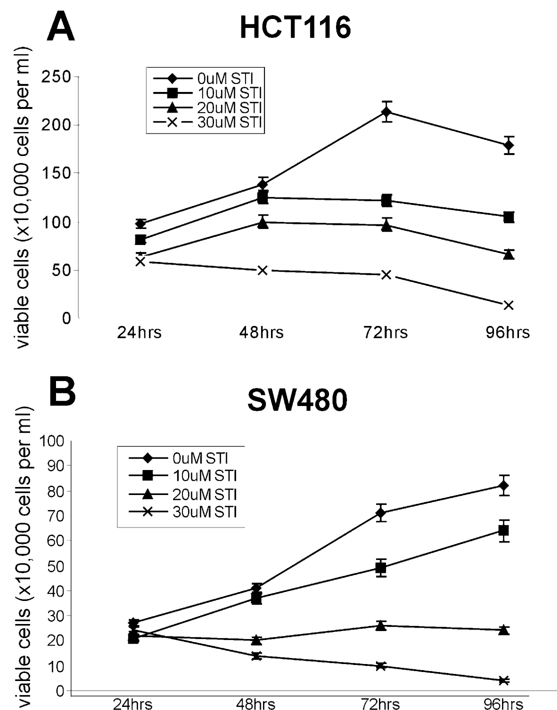

Fig. 3. STI-571 inhibits proliferation of human colon cancer cells. Exponentially growing HCT116 (A) and SW480 (B) cells were treated with STI-571 at the indicated concentrations. At 24, 48, 72, and 96 h after treatment, cells were collected by trypsinization. Viable cells were counted after Trypan Blue staining. Each assay condition was done in triplicate.

Gal4-responsive promoter), both of which were stably introduced into HEK 293 cells (designated as 293-GEM cells) and were shown to be tightly regulated by Wnt signal [42]. As shown in Fig. 4A, GFP expression in 293-GEM cells were readily detectable at as early as 24 h after stimulation with the Wnt1-conditioned medium. However, the Wnt1-CM stimulated GFP expression was significantly inhibited in the presence of 10 and 20 μM of STI-571 (Fig. 4B). These results corroborate well with the findings using the TCF4 reporter (Fig. 2C), strongly suggesting that STI-571 can effectively inhibit the β-catenin signaling activity. With activation of β-catenin, signaling has been reported in a broad range of human tumors, our encouraging findings may merit further pre-clinical and clinical investigations on the use of STI-571 as a (at least adjuvant) chemotherapeutic agent for human cancer.

4. Discussion

In this study, we sought to test whether tyrosine kinase inhibitor STI-571 could inhibit β-catenin signaling activity, and hence suppress cell proliferation. Using a β-catenin/TCF4-responsive reporter, we demonstrated that STI-571 could effectively inhibit the constitutive activity of β-catenin signaling in human colon cancer cell lines (i.e. HCT116 and SW480), as well as the Wnt1-induced activation of β-catenin signaling in HOS, HTB-94, and HEK 293 cells. Furthermore, using a concentration range comparable with several published studies [46–49], we presented evidence that STI-571 could effectively suppress the proliferation of human colon cancer cell lines HCT116 and SW480. Finally, we demonstrated that STI-571 could effectively inhibit the Wnt1-mediated activation of a GAL4-β-catenin heter-

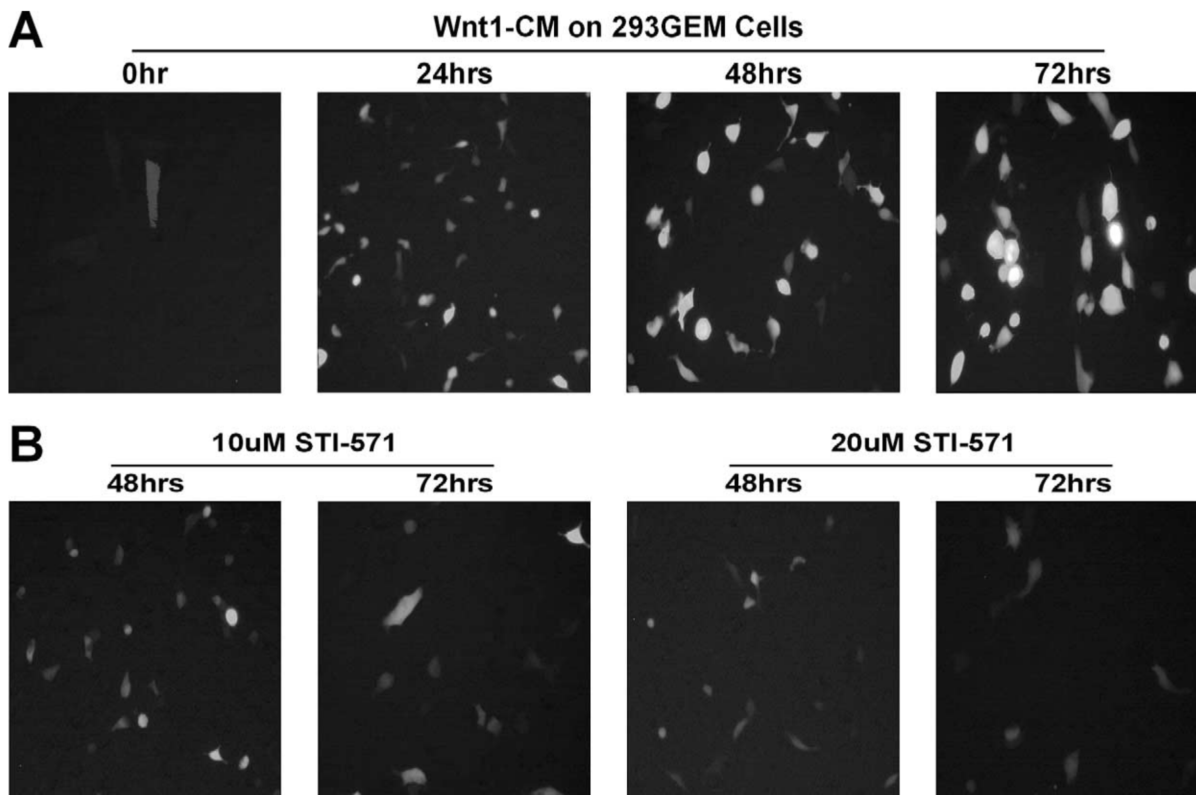

Fig. 4. STI-571 inhibits Wnt1-induced β-catenin activation in 293-GEM cells. Exponentially growing 293-GEM cells were exposed to Wnt1-conditioned medium (Wnt1-CM) in the absence (A) or presence (B) of STI-571 (at 10 or 20 μM). Signal intensity of green fluorescent protein was recorded using fluorescent microscopy at the indicated time after treatment.

ologous transcription system, namely the 293-GEM cells [42]. These findings suggest that tyrosine phosphorylation may play an important role in regulating β-catenin signaling activity.

Recent studies suggest that deregulation of β-catenin signaling may play an important role in human tumorigenesis [1,3]. With the exception of colon cancer, mechanisms underlying β-catenin deregulation are largely unknown in the vast majority of human tumors [12]. Several studies indicate that β-catenin can become a tyrosine-phosphorylated protein upon the activation of several protein tyrosine kinases, and β-catenin is an excellent substrate for protein tyrosine kinases, such as the Src kinase [50,51]. In fact, β-catenin is a major tyrosine-phosphorylated protein during mouse oocyte maturation and pre-implantation [52]. It has been reported that up-regulation of β-catenin tyrosine phosphorylation was more frequently observed in colon cancerous tissues than in the matching normal mucosa [53]. Several studies demonstrated that epidermal growth factor (EGF) is able to induce tyrosine phosphorylation of β-catenin, and in many cases, β-catenin is shown to associate with EGF receptor/c-erbB-2 gene product [54–62]. It has also been extensively documented that β-catenin protein becomes tyrosine-phosphorylated upon stimulation with hepatocyte growth factor (HGF)/scatter factor (SF) [31–33,63–64]. Moreover, β-catenin has been found in the complex containing HGF/SF receptor c-MET [63,65]. A recent study has demonstrated that oncogenic mutants of MET receptor tyrosine kinase can effectively activate the β-catenin signaling pathway. Other factors, including vascular endothelial growth factor (VEGF) [34–36], insulin-like growth factor 1 (IGF-1) [37,38], IGF-2 [39], and Gas6 (the ligand for the Axl RTK family) [40], have been shown to stimulate the tyrosine phosphorylation of β-catenin protein and to activate β-catenin signaling. Conversely, several forms of protein tyrosine phosphatases (PTPs), such as PTPμ and receptor PTPβ/ξ, have been shown to associate with β-catenin complex and to modulate tyrosine phosphorylation of the β-catenin protein [66–68]. The potential biological importance of the tyrosine-phosphorylated β-catenin remains to be defined. The current prevailing viewpoint is that tyrosine phosphorylation of β-catenin protein would result in its decreased association with E-cadherin, leading to the disruption of junctional assembly and an increased propensity for cancer cells to become invasive and metastasize [50,51,69]. However, growth factors that activate receptor protein tyrosine kinases have also been shown to stabilize cytoplasmic β-catenin protein and to activate downstream targets of the β-catenin signaling pathway [31–40], suggesting that tyrosine phosphorylation plays a role not only in regulating β-catenin's involvement in cell adhesion but also β-catenin signaling activity. Interestingly, it has been recently reported that phosphorylation of Tyr-654 at the C-terminal portion of β-catenin decreases its binding to E-cadherin and also stimulates association of β-catenin with the basal transcription machinery [70,71]. Thus, it would be interesting to elucidate the nodal point in which β-catenin/TCF4 signaling activity is regulated by tyrosine phosphorylation.

The introduction of STI-571 as an agent targeting the causative molecular event in CML has been heralded as a major advance in the treatment of cancer [22–24,72]. Although originally developed as an inhibitor of constitutively active Bcr-abl kinase, STI-571 is also able to inhibit Abl kinase, c-kit tyrosine kinase, and the platelet-derived growth factor (PDGF) receptor kinases [22,24]. It is conceivable that other tyrosine kinases may also serve as possible cellular targets of STI-571, especially when higher concentrations of STI-571 are used. Given the fact that deregulation of β-catenin is a frequent event in many types of human cancer [3], targeted inhibition of β-catenin signaling may represent an important alternative for the treatment of human cancer. Our results suggest that STI-571 could be used as a potential inhibitory agent of β-catenin signaling activity. Our findings are consistent with an early study, in which the combination of sulindac (a non-steroidal anti-inflammatory drug with established chemopreventive activity in colon cancer) and EKI-569 (an irreversible inhibitor of EGF receptor kinase) exhibited a pronounced chemopreventive effect in APC/Min$^{-/+}$ mice, a murine model of human familial adenomatous polyposis [73]. Furthermore, while we were preparing this report, Attoub et al. demonstrated that STI-571 was capable of inhibiting the proliferation of human colon cancer cell lines [49]. Thus, our findings corroborated well with these experimental results, and yet may provide a possible mechanistic explanation to the above observations. Taken together, our results suggest

that tyrosine phosphorylation may play an important role in regulating β-catenin signaling activity, and inhibition of this signaling pathway by STI-571 may be further explored as an important alternative/adjuvant treatment of human cancer.

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