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Tandutinib inhibits the Akt/mTOR signaling pathway to inhibit colon cancer growth

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Abstract

The c-Kit receptor can activate distinct signaling pathways including phosphatidylinositol-3-kinase (PI3K)/Akt and mammalian target of rapamycin (mTOR). Aberrant c-Kit activation protects cells from apoptosis and enhances invasion of colon carcinoma cells. Tandutinib is a novel quinazoline-based inhibitor of the type III receptor tyrosine kinases including c-Kit. We determined the effect of Tandutinib on colon cancer growth and identified a mechanism of action. Tandutinib inhibited phosphorylation of c-Kit, Akt, mTOR and p70S6 Kinase. In addition, Tandutinib significantly inhibited the proliferation and colony formation ability of colon cancer cell lines but did not affect normal colonic epithelial cells. There were increased levels of activated caspase 3 and Bax/Bcl2 ratio, coupled with a reduction in cyclin D1 suggesting apoptosis. There was also a downregulation of cyclooxygenase 2 (COX-2), vascular endothelial growth factor (VEGF) and interleukin-8 expression suggesting effects on cancer promoting genes. In addition, overexpressing constitutively active Akt partially suppressed Tandutinib-mediated colon cancer cell growth. *In vivo*, intraperitoneal administration of Tandutinib significantly suppressed growth of colon cancer tumor xenografts. There was a reduction in CD31-positive blood vessels, suggesting that there was an effect on angiogenesis. Tandutinib treatment also inhibited the expression of cancer promoting genes COX-2 and VEGF, and suppressed the activation of Akt/mTOR signaling proteins in the xenograft tissues. Together, these data suggest that Tandutinib is a novel potent therapeutic agent that can target the Akt/mTOR/p70S6K signaling pathway to inhibit tumor growth and angiogenesis.

Keywords

apoptosis; tumor xenograft; cancer promoting genes, cell cycle

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Introduction

Colorectal cancer is a second leading cause of adult cancer related death in the United States, and is associated with a high mortality rate (1). The lifetime risk of developing colorectal cancer in both men and women is about 1 in 20 (5.1%) (2). The American Cancer Society estimated 103,170 new cases (49,920 men and 53,250 women) would be diagnosed with colon cancer during 2012 (1, 3). Screening for colon cancer can be done by colonoscopy to find polyps, and removing these polyps at an early stage can prevent cancer progression. When the polyps are allowed to persist in the colon for a long time, they may develop into cancer. Chemotherapeutic compounds currently being used for the treatment of colorectal cancer include 5-fluorouracil, oxaliplatin and irinotecan (4). Because conventional therapies, including surgical resection, chemotherapy and radiation are often inadequate in treating this disease, new treatment options are critically needed. Despite the emergence of novel targeted agents and the use of various therapeutic combinations, no treatment options are available that are curative in patients with advanced cancer. The magnitude of this problem mandates the need for novel therapeutic agents.

Tandutinib (Fig. 1A) is a potent FLT3 antagonist of the piperazinyl quinazoline class that can also inhibit the activation of other class III receptor tyrosine kinases PDGFR and c-Kit (5). However, it does not have significant suppressive activity against a wide range of other kinases (5). By blocking FLT3 activation, Tandutinib suppresses the phosphorylation of Akt and Erk2 MAP kinase pathways thereby leading to induction of apoptosis in FLT3-ITD-positive human AML cell lines (5). Preclinical studies have demonstrated that Tandutinib is orally bioavailable, has a suitable pharmacokinetic and toxicity profile for clinical use, and is efficacious in murine models of FLT3-ITD-mediated disease (5, 6). In addition, Tandutinib also inhibits the receptor for macrophage-colony stimulating factor thereby affecting osteoclastogenesis and macrophage colony formation *in vitro* (7). Moreover, a recent study demonstrated that Tandutinib inhibits PDGFR-A activation resulting in reduced tumor cell growth and increased apoptosis *in vitro* in a sonic hedgehog-driven medulloblastoma mouse model (8). Since c-Kit expression is upregulated in colon cancers (9), we considered that Tandutinib might be novel therapeutic agent for the disease.

The c-Kit proto-oncogene encodes a transmembrane tyrosine kinase receptor. Aberrant c-Kit expression, described in subgroups of patients with colorectal cancer, correlates with dismal prognosis (10, 11). c-Kit mRNA expression has been detected in various human colonic adenocarcinoma cell lines (9, 12). Furthermore, activating mutations of c-Kit protects human colon adenocarcinoma cells against apoptosis and enhance their invasive potential (12). The c-Kit ligand stem cell factor (SCF) has been also detected in normal intestinal epithelial cells (13), suggesting autocrine and paracrine control of transforming functions by SCF in human colon cancer (9). Therefore, c-Kit is a therapeutic target for colorectal carcinoma.

Upon binding with its ligand stem cell factor SCF, c-Kit undergoes dimerization and autophosphorylation at specific tyrosine residues Tyr567 and Tyr719. This activated receptor then phosphorylates various signaling pathways the phosphatidylinositol 3-kinase (PI3K)/Akt/ mammalian target of rapamycin (mTOR)/ p70S6 kinase (p70S6K), Ras/

mitogen-activated protein kinase kinase (MEK)/mitogen-activated protein kinase (MAPK), the Janus kinase (JAK)/signal transducer and activator of transcription (STAT), and the Src signaling pathways (14). Activation of these pathways results in cellular transformation and differentiation through including proliferation, survival, adhesion, and chemotaxis (9). There is a positive feedback loop in that c-Kit can in turn be activated by the PI3K and MAPK pathway.

The PI3K/Akt/mTOR signaling axis plays a critical role in the proliferation, resistance to apoptosis, angiogenesis and metastasis that is central to the development and maintenance of colorectal cancers (15). PI3K is activated upon growth factors binding to their cognate receptors. Activated PI3K leads to the activation of Akt by phosphorylation at Ser473 and Thr308 (16). Akt activates several downstream targets including mTOR. Deregulation of mTOR signaling occurs in several human tumors including colon cancer (15). mTOR associates with Raptor (mTORC1 complex) to phosphorylate p70S6K, which in turn phosphorylates 4E-BP1, leading to increased cell proliferation (17). In addition, mTOR associates with Rictor (mTORC2 complex) and functions in a feedback loop to phosphorylate and activate Akt at Ser473 (16).

In this article, we are the first to demonstrate the effect of Tandutinib on colon cancer cells and have identified at least one mechanism of action to be through the inhibition of the Akt/mTOR signaling pathway.

Materials and Methods

Cells and reagents

HCT116, HT-29 and SW480 human cancer cells (all obtained from American Type Culture Collection, at passage 4) were grown in DMEM containing 10% heat inactivated fetal bovine serum (Sigma-Aldrich) and 1% antibiotic-antimycotic solution (Mediatech Inc) at 37°C in a humidified atmosphere containing 5% CO₂. Normal colon epithelial cells (FHC, CRL-1831) were grown in Ham's F12 medium 45%, Dulbecco's Modified Eagle's medium 45%, 25 mM HEPES, 10 ng/ml cholera toxin, 0.005 mg/ml insulin, 0.005 mg/ml transferrin, 100 ng/ml hydrocortisone, 10% fetal bovine serum (Sigma Aldrich) and 1% antibiotic-antimycotic solution (Mediatech Inc) at 37°C in a humidified atmosphere of 5% CO₂. All the cell lines used in this study were within 20 passages after receipt or resuscitation (~3 months of non-continuous culturing). The cell lines were not authenticated as they came from national repositories. Tandutinib was purchased from LKT Laboratories, St Paul, MN.

Proliferation and apoptosis assays

Cells were seeded on to 96 well plates and grown overnight. Then, the cells were treated with increasing doses of Tandutinib (0–50 µM) in 10% FBS containing DMEM. The cell proliferation was analyzed by hexosaminidase assay as described previously (18). For apoptosis, caspase 3/7 activity was measured using the Apo-one Homogeneous Caspase-3/7 Assay kit (Promega).

Colony formation assay

Briefly, 6 well dishes were seeded with 500 viable cells per well, treated with Tandutinib (25 μ M) in 10% FBS containing DMEM for 48 h, the Tandutinib-containing medium was removed, and the cells were incubated for an additional 10 days. Treatments were performed in triplicate. The colonies obtained were fixed in formalin and stained with Crystal violet. The colonies were compared with untreated cells.

Cell cycle analyses

Cells were treated with Tandutinib for 24- and 48-h and subsequently trypsinized and suspended in phosphate buffered saline (PBS). Single-cell suspensions were fixed using 70% ethanol for 2 h, and subsequently permeabilized with PBS containing 1 mg/ml propidium iodide (Sigma-Aldrich), 0.1% Triton X-100 (Sigma-Aldrich) and 2 μ g DNase-free RNase (Sigma-Aldrich) at room temperature. Flow cytometry was done with a FACSCalibur analyzer (Becton Dickinson) capturing 10,000 events for each sample. Histograms were analyzed for cell cycle compartments and the percentage of cells at each phase of the cell cycle was calculated using CellQuest (Becton Dickinson) analysis software.

Real time reverse-transcription polymerase chain reaction analysis

Total RNA isolated from HCT116 cells treated with Tandutinib using TRIZOL reagent and reverse transcribed with Superscript II reverse transcriptase in the presence of random hexanucleotide primers (Invitrogen). Complementary DNAs were then used for Real Time PCR using Jumpstart Taq DNA polymerase (Sigma-Aldrich) and SYBR Green nucleic acid stain (Molecular Probes). Crossing threshold values for individual genes were normalized to β -Actin. Changes in mRNA expression were expressed as fold change relative to control. Primers used in this study were as follows: β -actin: 5'-GCTGATCCACATCTGCTGG-3' and 5'-ATCATTGCTCCTCCTCAGCG-3'; COX-2: 5'-GAATCATTCACCAGGCAAATTG-3' and 5'-TCTGTACTGCGGGTGGAAACA-3'; VEGF: 5'-AGCGCAAGAAATCCCGGTA-3' and 5'-TGCTTTCTCCGCTCTGAGC-3'; IL-8: 5'-CTCTTGGCAGCCTTCCTGATT-3' and 5'-TATGCACTGACATCTAAGTTCCTTTAGCA-3'; cyclin D1: 5'-AATGACCCCGCACGATTTC-3' and 5'-TCAGGTTTCAGGCCTTGAC-3'. C-Myc: 5'-CACACATCAGCACAACTACGCA-3', and 5'-TTGACCCTCTTGGCAGCAG-3'.

Western blot analysis

Cell lysates were subjected to polyacrylamide gel electrophoresis and blotted onto Immobilon polyvinylidene difluoride membranes (Millipore). Antibodies were purchased from Cell Signaling Technology and Santa Cruz Biotechnology. The following are the catalog numbers for antibodies from Cell Signaling Technology: Capase-3-9662S, Bcl2-2876S, BclXL-2764S, Bax-5023S, cyclinD1-2926S, c-Myc-5605S, COX-2 -4842S, phosphor-c-Kit-3391S, c-Kit-3392S, phospho-Akt (Ser473)-9271S, phospho-Akt (Thr308)-9275S, Akt-9272S, phospho-mTOR-2974S, mTOR-2972S, phospho-p70S6 Kinase- 9208S, p70S6 Kinase-2708, phospho-4EBP1-2855S, 4EBP1-8594. The following are that catalog numbers for antibodies from Santa Cruz Biotechnology Inc: VEGF-sc-152,

IL-8-sc-7922, Actin-sc-1616. Specific proteins were detected by the enhanced chemiluminescence system (GE Healthcare).

Plasmids and Transfections

HCT116 and SW480 cells were transfected with plasmid 9008: 1036 pcDNA3 Myr-HA-Akt1 or the empty vector pcDNA3 (Addgene Inc, Cambridge, MA) and subsequently treated with 25 μ M Tandutinib for 48 h and performed western blot analysis. Cell proliferation and apoptosis were detected by hexoaminadase and the Apo-ONE Homogeneous Caspase-3/7 assays, respectively.

HCT-116 xenograft tumors in mice

Five-week-old male athymic nude mice (Charles River Laboratory) were utilized for *in vivo* experiments. They were maintained with water and standard mouse chow *ad libidum* and used in protocols approved by the University's Animal Studies Committee. Animals were injected with 5 $\times$ 10⁶ HCT116 cells in the left and right flank and allowed to form xenograft. One week following implantation, and after observing the presence of a palpable tumor, Tandutinib (50 mg/kg body weight) was administered intraperitoneally daily for 21 d. Tumors were measured weekly. At the end of treatment the animals were euthanized, and the tumors were removed and weighed for use in histology, immunohistochemistry, and gene expression studies.

Immunohistochemistry

Tissues embedded in paraffin were cut to a section of 4 μ M, deparaffinized and treated with antigen decloaker for 15 minutes (Biocare Medical). Then they were blocked with Avidin/Biotin for 20 min (Invitrogen). The slides were incubated with anti-COX-2 (sc-1746), anti-VEGF (sc-152), anti-cyclin D1 (sc-753), anti-CD31 (ab28364), anti-pAkt (9271S), anti-pmTOR (2976S), anti-pP70 S6 Kinase (9208S) and anti-p4EBP1 (2855S) antibodies for overnight at 4 °C. Next the slide was treated with broad-spectrum secondary antibody (Invitrogen) and HRP- conjugate for one hour and then developed with DAB (Invitrogen). Finally, the slides were counterstained with hematoxylin and mounted. The slides were examined in Nikon Eclipse Ti microscope under a 40 $\times$ objective.

Statistical analysis

All values are expressed as the mean $\pm$ SEM. Data was analyzed using an unpaired 2-tailed t test. A *P* value of less than 0.05 was considered statistically significant.

Results

Tandutinib inhibits colon cancer cell proliferation

Previous studies have demonstrated that Tandutinib blocks growth of leukemia cells (5). In order to determine whether Tandutinib affects colon cancer cells, we first determined the effect of the compound on the proliferation of three different colon cancer cell lines and normal colon epithelial cells. Tandutinib significantly suppressed proliferation of HCT116, SW480 and HT-29 cells in a dose and time dependent manner (Fig. 1B). This anti-

proliferation effect on tumor cells was seen within 24 h at a dose of 25 μ M, which continued to significantly increase over the next 72 h (Fig. 1B). In contrast, Tandutinib did not affect proliferation of FHC normal colon cells even at doses of 30 μ M (Fig. 1C). To determine the long-term effect of Tandutinib treatment, cells were treated with Tandutinib for 48 h before it was allowed to grow in normal medium. There were lower numbers of colonies in all three colon cancer lines following drug treatment when compared to untreated control (Fig. 1D). These data suggest that Tandutinib treatment suppresses growth of colon cancer cells in culture.

Tandutinib induces apoptosis

Given the effects of Tandutinib on suppression of proliferation and colony formation, we next determined whether the compound affects cell cycle progression. After 24 h of Tandutinib treatment, there was significantly higher number of cells arrested in the G2/M phase (Fig. 2A). In addition, there were a greater number of dead cells in both HCT116 and SW480 cell lines (Fig. 2A). At 48 h, while Tandutinib induced G0/G1 arrest in HCT116 cells there was G2/M arrest in SW480 cells. We also determined how the cells died following Tandutinib treatment. Caspases are a family of proteins known for the execution of apoptosis. Caspase-3 and 7 are key effector molecules of apoptosis in variety of cancer cells that amplify signals from initiator caspases, such as caspase 8 or caspase 9 (19). Increased caspase 3/7 activity was observed within 48 h in all three cell lines HCT116, SW480 and HT-29 treated with Tandutinib suggesting the induction of apoptosis (Fig. 2B). This was further confirmed by western blot analyses where treatment with Tandutinib demonstrated a significant increase in the levels of cleaved caspase-3 (Fig. 2C). In addition, Tandutinib inhibited the expression of anti-apoptotic genes Bcl2 and Bcl-XL proteins while increasing the expression of apoptosis-promoting Bax protein (Fig. 2D). Moreover, we have also determined the effect of Tandutinib on caspase 3/7 activity in FHC normal colonic epithelial cells. There was no significant increase in caspase 3/7 activity in normal colonic epithelial treated with 25 μ M of Tandutinib (data not shown). These data suggest that Tandutinib is a potent inducer of apoptosis of colon cancer cells.

Tandutinib affects expression of cell cycle related proteins

To further characterize the mechanism of cell death, we examined the level of expression of several known cell cycle regulatory factors (20). Cyclin D1 overexpression has been linked to the development and progression of cancer (21). It is a cell cycle regulatory protein that regulates the G1 to S-phase transition of the cell cycle and functions as a cofactor for several transcription factors. Consistent with cell cycle arrest, the expression of cyclin D1 was decreased at both the mRNA and protein levels in the two cell lines (Fig. 3A and B). Furthermore, cyclin A1 and B1 also reduced following Tandutinib treatment in both HCT116 and SW480 cells (Fig. 3B). In addition, c-Myc is upregulated in cancers. In both HCT116 and SW480 cells, Tandutinib treatment resulted in reduced levels of c-Myc expression (Fig. 3A and B).

Tandutinib inhibits expression of cancer-promoting genes

Cyclooxygenase-2, a key rate-limiting enzyme in prostaglandin synthesis is overexpressed in many cancers. Previous studies have demonstrated increased COX-2 levels in colorectal

cancer (22, 23). COX-2 plays a significant role in increased invasiveness, promotion of angiogenesis and resistance to apoptosis (24). Therefore, we next determined the effect of Tandutinib on COX-2 expression. Tandutinib reduced COX-2 mRNA and protein levels in both HCT116 and SW480 cells (Fig. 3C and D). Prostaglandins and the other tumor promoters are known to induce the expression of vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8) in epithelial cells, thereby promoting angiogenesis and hence tumor growth (25). VEGF and IL-8 are known potent inducers of capillary growth into the tumor, and without angiogenesis, tumor growth normally stops at a diameter of about 1 to 2 mm (26). Hence, we also determined the effect of Tandutinib on the expression of these two genes. Both VEGF mRNA and IL-8 protein expression were significantly reduced in both HCT116 and SW480 cells (Fig. 3C and D). Similar results were obtained in HT-29 cells (data not shown).

Tandutinib affects c-Kit and Akt/mTOR signaling

Tandutinib is a known inhibitor of PDGFR and c-Kit in AML cells lines (5). Aberrant activation of c-Kit protects colon carcinoma cells from apoptosis and enhances their invasive potential (12). To further demonstrate the effect of Tandutinib on c-Kit in colon cancer cells, a western blot analysis was carried out. Tandutinib treatment significantly downregulated the phosphorylation of c-Kit in both HCT116 and SW480 cells (Fig. 4A). Similar result was obtained in HT29 cells (data not shown). Previous studies have demonstrated that Tandutinib selectively blocks FLT3-ITD phosphorylation, leading to an inhibition of Akt and Erk signaling and to induction of apoptosis in FLT3-ITD-positive human AML cell lines (5). To determine whether a similar mechanism was occurring in colon cancer cells, we determined the effect of Tandutinib on the Akt/mTOR pathway. Western blot analyses demonstrated that Tandutinib treatment downregulated the phosphorylation of both Akt and mTOR in the two cell lines (Fig. 4B). Furthermore, Tandutinib also further downregulated the phosphorylation of downstream target protein P70S6Kinase and 4EBP1 (Fig. 4B). In addition, Tandutinib treatment increased PTEN expression in both HCT116 and SW480 cells. We also determined the effect of Tandutinib on Akt phosphorylation in normal colonic epithelial cells. There was no significant difference observed in the phosphorylation of Akt in normal FHC cells (data not shown).

We next determined the effect of overexpressing a constitutively active form of Akt on Tandutinib mediated cell growth. We overexpressed myristoylated Akt in the two cell lines, HCT116 and SW480, and subsequently treated the cells with Tandutinib. As before, in control cells, Tandutinib treatment significantly suppressed cell proliferation. However, this Tandutinib mediated suppression was reduced in the presence of myristoylated Akt (Fig. 4C right panel). We confirmed this by determining the levels of apoptosis by caspase 3/7 assay. While Tandutinib alone increased caspase 3/7 activity, this was significantly reduced in cells expressing constitutively active Akt in both HCT116 and SW480 cells (Fig. 4C left panel). Additional confirmation was obtained by western blot analyses. Expression of constitutively active Akt resulted in increased expression of Bcl2 and reduced expression of Bax proteins, even in the presence of Tandutinib in the two cell lines (Fig. 4D right panel). These data suggest that Tandutinib-mediated suppression of cell growth occurs in part through suppression of Akt phosphorylation.

Tandutinib inhibits colon tumor xenograft growth and angiogenesis

To evaluate the role of Tandutinib on tumor growth *in vivo*, we next examined its effects on HCT116 colon cancer cell xenografts. HCT116 were injected into the flanks of athymic nude mice and allowed to develop into a xenograft. One week following injection of the cells Tandutinib mixed with 5% sodium bicarbonate was administered intraperitoneally daily for three weeks. Tandutinib inhibited the growth of the tumor xenografts (Fig. 5C). The excised tumors from control animals weighed ~1200 mg, while those treated with Tandutinib weighed ~400 mg (Fig. 5B top and bottom panel). In addition, tumor volume was significantly decreased (Fig. 5C). There was no apparent change in liver, spleen, or body weight in the animals (data not shown). These data imply that the Tandutinib is a potential therapeutic agent for treating colon cancers. We also determined the effect of the combination on tumor vascularization by staining for endothelial-specific antigen CD31. As shown in Fig. 5 D, the treatment with the Tandutinib leads to a significant reduction in CD31 staining and to the obliteration of the normal vasculature (Fig. 5D).

Tandutinib inhibits the expression of cancer promoting genes and Akt/mTOR signaling proteins in tumor xenograft tissues

Given that Tandutinib inhibits cancer-promoting genes COX-2 and VEGF in colon cancer cells and reduced microvasculature within the xenograft tumors, we investigated whether the treatment affects the COX-2 and VEGF in the tissues. Western blot analyses demonstrated that the Tandutinib significantly reduced the expression of COX-2 and VEGF when compared to controls in the tumor xenograft tissues (Fig. 6A). Cyclin D1 protein levels were also significantly lower in Tandutinib treated tumor xenografts (Fig. 6A). These data were confirmed by immunohistochemistry analyses (Fig. 6C, Left panel). We also quantified the number of cells expressing COX-2, VEGF and cyclin D1 in the immunohistochemistry and found all three proteins to be significantly decreased following Tandutinib treatment (Fig. 6D, Left panel). These data suggest that Tandutinib affects cancer- promoting genes *in vivo*.

We also examined the effects on Akt/mTOR signaling proteins in the tumor tissues obtained from control and Tandutinib treated mice. Treatment with Tandutinib again resulted in significantly lower levels of phosphorylation of Akt, mTOR, P70S6 Kinase and 4E-BP1 when compared to control untreated tumors (Fig. 6B). Again, further confirmation of the down regulation was obtained by immunohistochemistry for the proteins in the xenograft tissues (Fig. 6C Middle and Right Panel). We also determined the number of cells expressing phospho-Akt, phospho-mTOR, phospho-P70 S6 Kinase and phospho-4EBP1 in the tumor sections and found them to be significantly decreased following Tandutinib treatment (Fig. 6D Right panel). These data suggest that Tandutinib significantly affects expression of Akt/mTOR signaling-related proteins, which might contribute to the inhibitory effects of this treatment.

Discussion

Given the high mortality rate due to colon cancer and the significant morbidity, apparent toxicity and poor response rates of current chemotherapy regimens there has been a big push to identify novel therapeutic modalities that have fewer toxicity profiles. The PI3K/Akt/

mTOR signaling axis plays a critical role in the proliferation, resistance to apoptosis, angiogenesis and metastasis that is central to the development and maintenance of colorectal cancer cells (27–29). Therefore, therapeutic targeting of the PI3K pathway with its downstream targets Akt and mTOR at multiple molecular levels may provide better antitumor effects than selective inhibition of only one component of the pathway. Our results indicate that Tandutinib possesses potential as a promising therapeutic agent against colon cancer.

Previous studies demonstrated that c-kit over-expression in colorectal cancer patients resulted in poor prognosis (10, 11). C-kit plays an important role in cell growth differentiation, motility and cell survival in a variety of cancers including colorectal cancer (9). Furthermore, c-kit protects the cells from undergoing apoptosis while also increasing their invasiveness in colon cancer cells (12). In fact, the c-kit tyrosine kinase inhibitor STI571 significantly inhibits cells survival and invasive potential of these cells, and hence may be appropriate for colorectal cancer therapy (9, 30). Another study also reported that BAY 43-9006 inhibits c-Kit, along with several receptor tyrosine kinases. Moreover, BAY 43-9006 inhibits tumor growth, angiogenesis and MAPK pathway in colon, pancreatic, and breast tumor cell lines, in part through inhibiting the expression of VEGFR-2, and -3 (31). In addition, c-Kit receptor signaling enhanced the proliferation and invasion of colorectal cancer cells through the PI3K/Akt pathway (32). Similar to these studies, Tandutinib treatment effectively down regulated the c-kit phosphorylation in colon cancer cells. Moreover, Tandutinib inhibited cell proliferation and induced apoptosis in colon cancer cells in part through inhibiting PI3K/Akt signaling.

Aberrant PI3K/Akt signaling is common in colon cancer. Recent studies have reported the potential for therapeutic targeting of the PI3K/Akt/mTOR network at multiple molecular levels (33, 34). mTOR is a serine/threonine kinase that is activated in response to various stimuli through PI3K/Akt (35). mTOR resides in 2 distinct multiprotein complexes called mTORC1 and mTORC2 (36). mTORC1 directly phosphorylates ribosomal protein S6 kinase 1 (S6K1) and the eukaryotic translation initiation factor eIF4E-binding protein 1 (4EBP1), both involved in protein translation (37, 38). Moreover, mTORC2 can phosphorylate Akt on Ser 473, thereby leading to activation of the Akt pathway and thus several cancer-related cellular responses, including increased cell growth, proliferation, and survival (39). A recent preclinical study with OSI-027, a potent inhibitor of Akt/mTOR suppressed the growth of colon cancer (39). In addition, Akt and mTOR inhibitors can downregulate leptin mediated PI3K/Akt/mTOR signaling affecting colon cancer cell proliferation and inducing apoptosis (40). Our studies in this manuscript had similar results with Tandutinib effectively downregulating phosphorylation of both Akt and mTOR phosphorylation and also mTOR downstream target proteins phospho-p70 S6K (Thr389) and phospho-4EBP1 (Thr37/46). These findings suggest that Tandutinib downregulates the functions of Akt and mTOR. Moreover, Tandutinib inhibited proliferation and induced apoptosis in colon cancer cell lines, and suppressed formation of microvessels in the xenografts indicating that the *in vivo* anti-cancer effects of Tandutinib may be due to induction of apoptosis, in addition it also inhibits angiogenesis.

In our *in vivo* studies, we observed marked decrease in tumor xenograft growth in mice treated with Tandutinib. Moreover, there was no apparent toxicity observed in liver, kidney, and spleen. A significant body of evidence suggest that constitutive COX-2 expression is a contributing factor for promoting colorectal cancer (41). In addition, there are various genetic, epigenetic, and inflammatory pathways involved in the etiology and development of colorectal cancer (41). Tandutinib treatment seems to mediate its actions through multiple molecular targets, including COX-2. Given the high levels of resistance to apoptosis due to COX-2 overexpression (42, 43), treating cells with Tandutinib could potentially restore apoptotic susceptibility of colon cancer cells in part through the downregulation of this gene.

Recent studies showed that IL-8 and its receptors are significantly upregulated in the tumor and act as key regulators of proliferation, angiogenesis, and metastasis. Although IL-8 overexpression has been correlated with the inflammation-related risk of sporadic colorectal cancer (44), it is also a potent inducer of proliferation of colorectal cancer cells (45). Hence, IL-8 has been considered a therapeutic target (46). In this regard, the IL-8 receptor antagonist SCH-527123 was shown to inhibit tumor growth and sensitize cells to oxaliplatin in preclinical colon cancer models (47). Tandutinib treatment inhibits IL-8 expression but it remains to be seen if this inhibition also correlates with changes in inflammatory responses that are associated with the development of cancer.

VEGF and its receptors are overexpressed in colon cancer and play an important in angiogenesis and promotion of tumor growth (48, 49). Pharmacological inhibition with STI571 (Gleevec®) of VEGF expression also suppresses VEGF and bFGF induced angiogenesis *in vivo* (50). The compound also inhibits angiogenesis and tumor growth in an experimental bone metastasis model (50). Our studies with Tandutinib significantly downregulating VEGF expression in both *in vitro* and *in vivo* tumor tissues suggest that Tandutinib can also affect angiogenesis. Future studies are warranted to determine whether the combination of Tandutinib with known angiogenesis inhibitors such as STI571 has greater effect than either compound alone.

In conclusion, our studies show that treatment of colon cancer cells with Tandutinib results in significant inhibition of tumor cell growth, both *in vitro* and *in vivo*. Our studies show that Tandutinib affects Akt/mTOR and c-Kit pathways. One of the biggest problems with therapeutic compounds is the toxicity to normal cells. One mechanism to reduce toxicity is to use a combination of compounds that have synergistic activity. Hence, identifying a compound or compounds that can act synergistically with Tandutinib, may have value in reducing toxicity associated with the amount of compound needed to have therapeutic efficacy. Nevertheless, in our studies we observed that Tandutinib does not affect proliferation of normal colonic epithelial cells, strongly suggesting that the compound has promising potential for use as a therapeutic option for colon cancer.

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Figure 1. Tandutinib inhibits colon cancer cell proliferation

A, Topological structure of Tandutinib. B, Proliferation of colon cancer cells following Tandutinib treatment. Colon cancer cells were incubated with increasing doses of Tandutinib (0–50 μ M), and cell proliferation was measured at three different time points up to 72 h. Tandutinib treatment resulted in a significant dose- and time-dependent decrease in cell proliferation in all three cell lines when compared with controls. C, Proliferation of human normal colonic epithelial cells is not affected by Tandutinib. FHC cells were

incubated with 30 μ M Tandutinib for 48 h and analyzed for cell proliferation. Data shows that Tandutinib does not affect proliferation of the normal colonic epithelial cells. D, Tandutinib inhibits colony formation. Cells were incubated with 25 μ M Tandutinib for 48 h. Following this, the cells were allowed to grow and form colonies. Tandutinib inhibits colony formation. Results are representative of three independent experiments.

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Figure 2. Tandutinib induces cell death by apoptosis

A, Cell cycle analysis of Tandutinib-treated cells. HCT116 and SW480 cells were treated with 25 μ M of Tandutinib for 24- and 48 h and then examined by flow cytometry following propidium iodide staining for DNA content. Tandutinib treatment induced G₂/M arrest at 24 h. At 48 h, the compound induced cell death. Graphs are representative of data collected from three experiments. B, Tandutinib induces caspase 3/7 activity. HCT116, SW480 and HT-29 cells were treated with 25 μ M Tandutinib for 48 h and tested for Caspase 3/7 activity. Tandutinib treatment induces apoptosis in all the three cell lines, when compared to control

cells (* $p < 0.05$). C, Tandutinib induces caspase 3, an apoptosis mediator. Lysates of cells treated with 25 μ M Tandutinib for 48 h were analyzed by western blotting for caspase 3 protein. Tandutinib treatment resulted in increased levels of cleaved (activated) caspase 3 when compared to control. D, Lysates from Tandutinib treated cells was analyzed by western blotting for Bax and Bcl-2 proteins. Tandutinib treatment induces proapoptotic protein Bax and reduces expression of anti-apoptotic protein Bcl-2 family proteins when compared with control.

Figure 3. Tandutinib affects expression of cell cycle related proteins and cancer-promoting genes
 A, Real Time PCR analyses for cell cycle genes. Total RNA from HCT116 cells treated with 25 μ M of Tandutinib for 48 h was subjected to real Time PCR analyses. Tandutinib treatment significantly reduced the expression of cyclin D1 and c-Myc mRNA (* $p < 0.05$). B, Western blotting for cell cycle genes. Lysates from HCT116 and SW480 cells were incubated with 25 μ M of Tandutinib and analyzed by western blotting for cyclin A1, B1, D1 and c-Myc proteins. Expression of all four proteins was suppressed following Tandutinib treatment. C, Real Time PCR analyses for tumor promoting genes. Total RNA subjected to

Real Time RT-PCR analyses showed that Tandutinib treatment significant reduced the expression of COX-2, VEGF and IL-8 mRNA (* $p < 0.05$). D, Western Blotting for tumor promoting genes. Tandutinib treatment suppressed the levels of cancer promoting genes COX-2, VEGF and IL-8.

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Figure 4. Tandutinib affects c-Kit and Akt/mTOR signaling pathway in colon cancer cells
 A, Western blotting for c-Kit. Lysates from HCT116 and SW480 cells incubated with 25 μ M of Tandutinib were analyzed by western blotting for phosphorylation of c-Kit levels. Tandutinib treatment significantly suppressed phosphorylation of c-Kit protein. B, Western Blotting for mTOR pathway factors. Lysates from HCT116 and SW480 cells were analyzed by western blotting for phosphorylation of Akt, mTOR, p70S6Kinase, 4EBP1 levels and PTEN proteins. Tandutinib treatment significantly reduced the phosphorylation of Akt, mTOR, p70S6Kinase, and 4EBP1, but increased PTEN expression. C, (Left panel),

Proliferation of cells following overexpression of constitutively active form of Akt. In the presence of constitutively active Akt, Tandutinib effect of suppressing cell proliferation is significantly reduced. (Right panel), Caspase 3/7 activity. In the presence of constitutively active Akt, Tandutinib effect of inducing caspase 3/7 activity is significantly reduced. * $P < 0.05$. D. Western Blotting for overexpressed constitutively active form of Akt, Bcl2 and Bax. Lysates from HCT116 and SW480 cells were analyzed by western blotting for Akt phosphorylation in both HCT116 and SW480 cells (Left panel). Akt phosphorylation and Bcl2 levels are high, even in the presence of Tandutinib. In contrast, Bax protein levels remain low (Right panel).

Figure 5. Tandutinib inhibits growth of colon cancer xenografts

A, Experimental plan, HCT116 cells were injected in to the flanks of nude mice and palpable tumors were allowed to develop for 7 d. Subsequently, Tandutinib (50 mg/kg bw) was injected daily intraperitoneally every day for 21 d. Tumor size was measured every week. On day 22, tumors were excised and subject to further analyses. B, Tandutinib treatment resulted in significantly lower tumor weight when compared to control. There was a significant reduction in tumor size in Tandutinib treated animals when compared to controls (* $p < 0.05$) (top panel). Control and Tandutinib treated tumor photos (bottom panel).

C, Tumor volumes in Tandutinib treated mice were smaller when compared to control (*P<0.05). D, Tumor sections were stained for CD31, an endothelial cell specific surface marker. There was significant reduction in the number of microvessels. A representative figure is presented showing significant reduction in microvessels (400 ×).

Figure 6. Tandutinib suppresses expression of cancer promoting genes and activation of Akt/mTOR pathway in tumor xenografts

A, Western blot analyses show that tissue lysates from the Tandutinib treated animals tumor have significantly lower levels of COX-2, VEGF and cyclin D1 expression. B, Western blot analyses demonstrate that tissue lysates from the Tandutinib treated animals tumor have significantly lower levels of phosphorylation in Akt, mTOR, p70S6Kinase and 4EBP1 levels. C, Immunohistochemistry shows that treatment with Tandutinib reduced the expression of COX-2, VEGF and cyclin D1 (Left panel). Immunohistochemistry shows that treatment with Tandutinib reduced the phosphorylation in Akt, mTOR, p70S6Kinase and

4EBP1 in the tumor xenograft tissues. Representative photographs are the magnification of $\times 400$ (Middle and Right panel). D, Immunohistochemistry quantification shows that treatment with Tandutinib reduced the expression of COX-2, VEGF and cyclin D1 positive cells (* $p < 0.05$) (Left panel). Quantification shows that treatment with Tandutinib reduced the phosphorylation in Akt, mTOR, p70S6Kinase and 4EBP1 positive cells (* $p < 0.05$) (Right panel).