Inhibition of STAT3 Signaling Pathway by Nitidine Chloride Suppressed the Angiogenesis and Growth of Human Gastric Cancer

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Abstract

STAT3 has been strongly implicated in human malignancies, and constitutive activation of STAT3 serves a crucial role in cell survival, angiogenesis, immune evasion, and inflammation. In this study, we showed that nitidine chloride, a natural phytochemical alkaloid derived from *Zanthoxylum nitidum* (Roxb) DC, exerts potent anticancer activity through STAT3 signaling cascade. Nitidine chloride dose dependently suppressed VEGF-induced endothelial cell proliferation, migration, and tubular structure formation *in vitro* and dramatically reduced VEGF-triggered neovascularization in mouse cornea and Matrigel plugs *in vivo*. This angiogenesis inhibition mediated by nitidine chloride was well interpreted by the suppression of Janus kinase 2/STAT3 signaling and STAT3 DNA-binding activity in endothelial cells. Furthermore, nitidine chloride suppressed the constitutively activated STAT3 protein, its DNA-binding activity, and the expression of STAT3-dependent target genes, including cyclin D1, Bcl-xL, and VEGF in human gastric cancer cells. Consistent with the earlier findings, nitidine chloride inhibited gastric tumor cell growth and induced tumor cell apoptosis *in vitro* and effectively suppressed the volume, weight, and microvessel density of human SGC-7901 gastric solid tumors (*n* = 8) at a dosage of 7 mg/kg/d (intraperitoneal injection). Immunohistochemistry and Western blot analysis further revealed that the expression of STAT3, CD31, and VEGF protein in xenografts was remarkably decreased by the alkaloid. Taken together, we propose that nitidine chloride is a promising anticancer drug candidate as a potent STAT3 signaling inhibitor. *Mol Cancer Ther;* 11(2); 277–87. ©2011 AACR.

Introduction

Gastric cancer is one of the most common digestive malignancies in the world (1). Despite considerable improvements achieved through systemic therapy, the treatment of gastric cancer especially remains extremely unfavorable, with a 5-year survival rate of only 10% to 15% (2). Limited effects of conventional chemotherapy drugs including capcitabine, 5-fluorouracil, and paclitaxel on patient survival rate underscores the need for new strategies to inhibit gastric cancer growth. Gastric tumors can trigger substantial development of new blood vessels in a process called angiogenesis to nourish tumor growth (3). Angiogenesis is a rate-limiting process including the destabilization of integrated blood vessel, endothelial cell proliferation, migration, and tubulogenesis. Numerous reports have shown that disrupting tumor angiogenesis effectively suppresses tumor growth and metastasis (4). As a potential target, the STAT3, has been shown to be highly expressed in gastric cancer and be strongly linked to tumor angiogenesis and metastasis (5). Therefore, searching for novel STAT3-targeted antiangiogenic agents is urgently needed for all cancer treatments, including gastric cancer.

STAT3, a latent self-signaling transcription factor, is activated by certain interleukins (e.g., IL-6) and growth factors. Constitutive and aberrant activation of STAT3 occurs at a frequency of 50% to 90% in a broad range of human malignancies, suggesting that STAT3 pathway is significantly associated with tumor VEGF overproduction (6). Furthermore, recent studies have identified STAT3 as a direct transcriptional activator of VEGF and hypoxia-inducible factor (HIF)-1α under hypoxia (7), which are key stimuli known to initiate endothelial cell migration and differentiation. Upon activation, STAT3 undergoes phosphorylation, homodimerization, nuclear translocation, and DNA binding, which subsequently leads to transcription of various target genes, such as cyclin D1,
Bcl-2, Bcl-xL, matrix metalloproteinase 2 (MMP2), and VEGF, to regulate cell survival, angiogenesis, immune evasion, and inflammation in tumor microenvironment (8–10). Interfering with activated STAT3 signaling contributes to angiogenesis inhibition, tumor growth arrest, and metastasis suppression (11, 12). Currently, STAT3 inhibitor, including natural compounds, peptide, peptidomimetic compounds, small molecules, and oligonucleotides, have been developed and are undergoing into clinical settings (4, 13, 14). Thus, agents that suppress STAT3 activation are promising for prevention and treatment of cancer.

Nitidine chloride (Fig. 1A), a natural bioactive phytochemical alkaloid derived from Zanthoxylum nitidum (Roxb) DC, was initially reported to have significant antioxidant, antifungal, anti-inflammatory, and analgesic bioactivities (15). Subsequent studies proved that nitidine chloride exhibited anticancer potential through the induction of apoptosis in a wide variety of human tumor cell lines, such as lung, breast, liver, oral, and osteosarcoma in vitro (16). Activation of caspase-3 (17), inhibition of topoisomerase y (18), and suppression of human immunodeficiency virus reverse transcriptase (19) have been implicated in niditine-mediated anticancer and immunomodulatory function. However, the precise molecular target and underlying mechanisms of its antitumor efficacy are poorly defined to date.

Considering the critical roles of STAT3 signaling in the angiogenic and neoplastic process (20, 21), we screened a number of natural compounds and found that nitidine chloride exerted its antiangiogenic and antitumor property through the suppression of STAT3 pathway. We provide evidence that nitidine chloride dose dependently suppresses the activation of STAT3, its DNA-binding activity, and its transcriptional activity in both human endothelial cells and gastric cancer cells. As a result, nitidine chloride effectively inhibited tumor angiogenesis and tumor growth in an experimental gastric cancer xenograft mouse model. Therefore, our findings indicate that nitidine chloride is a promising candidate compound that can be further optimized to be a therapeutic agent for gastric cancer.

Figure 1. Nitidine chloride inhibits VEGF-induced endothelial cell proliferation, migration, and tubular-structures formation in vitro. A, the chemical structure of nitidine chloride (NC). B, nitidine chloride significantly inhibited cell proliferation dose dependently. Cell viability was determined by MTS assay. C, nitidine chloride remarkably inhibited VEGF-induced endothelial cells migration. D, nitidine chloride remarkably inhibited the tube formation of endothelial cells. Columns, mean from 3 independent experiments with triplicate; bars, SD; **, P < 0.01 versus VEGF alone group.
Materials and Methods

Reagents
Nitidine chloride (purity >98%) was purchased from Shanghai Winherb Medical Science. A 50 mmol/L stock solution was prepared in dimethyl sulfoxide (DMSO; Sigma), stored at −20°C and then diluted as needed in cell culture medium. Recombinant human VEGF (VEGF165) was a gift from the Experimental Branch of the NIH (Bethesda, MD). Growth factor–reduced Matrigel (Becton Dickinson) was used as basement membrane matrices. A 50 mmol/L stock solution of nitidine chloride (Sigma) was prepared in dimethyl sulfoxide (DMSO; Sigma), stored at −20°C and then diluted as needed in cell culture medium.

Cell culture
Primary human umbilical vein endothelial cells (HUVECs) were a gift from Dr. Xinli Wang (Cardiothoracic Surgery Division of Michael E. DeBakey Department of Surgery, Baylor College of Medicine, Houston, TX) and cultured in endothelial cell culture medium (ECM) as described previously (22). HUVECs were confirmed by their typical microscopic morphology: homogeneous, large, polygonal, and cobblestone like. Human gastric cancer AGS cells were purchased from American Type Culture Collection, and SGC-7901, MGC-803, and BGC-823 cancer cell lines were obtained from the China Center for Type Culture Collection, and SGC-7901, MGC-803, and BGC-823 cancer cell lines were obtained from the China Center for Type Culture Collection, and SGC-7901, MGC-803, and BGC-823 cancer cell lines were obtained from the China Center for Type Culture Collection, and SGC-7901, MGC-803, and BGC-823 cancer cell lines were obtained from the China Center for Type Culture Collection, and SGC-7901, MGC-803, and BGC-823 cancer cell lines were obtained from the China Center for Type Culture Collection, and SGC-7901, MGC-803, and BGC-823 cancer cell lines were obtained from the China Center for Type Culture Collection. Gastric cancer cells were cultured in RPMI-1640 medium supplemented with 40% FBS (HyClone Laboratories). All the above cells were maintained at 37°C, 5% CO2.

Cell viability assay
Briefly, HUVECs (3 × 104 to 4 × 104 cells per well) were seeded in 96-well plates, exposed to various concentrations of nitidine chloride and with VEGF (50 ng/mL) for 1 hour and then seeded onto the Matrigel layer in 48-well plates at a density of 5 × 104 to 1 × 105 cells per well. After incubation for 6 to 8 hours, angio-geneses were assessed on the basis of formation of capillary-like structures. Tubes in randomly chosen microscopic fields were photographed (Olympus; original magnification, ×100). Three independent experiments were carried out.

Animal studies
CS7/BL/6 and nude mice used in our present study were purchased from National Rodent Laboratory Animal Resources (Shanghai, China) and maintained according to the NIH standards established in the “Guidelines for the Care and Use of Experimental Animals.” All of the experimental protocols were approved by the Animal Investigation Committee of East China Normal University.

Matrigel plug assay
As described elsewhere (24), 0.5 mL of Matrigel in the presence or absence of 100 ng VEGF and 20 units of heparin, plus indicated amount of nitidine chloride (30 and 60 μg) was subcutaneously injected into the ventral area of CS7/BL/6 mice (n = 4–6). Seven days after the implantation, intact Matrigel plugs were carefully removed. Those plugs were then fixed and embedded in paraffin. Specific blood vessel staining with CD31 antibody was carried out on the 5-μm sections according to the protocol. Microphotographs were taken by Leica DM 4000B photomicroscope (magnification, ×400).

Mouse corneal micropocket assay
The modified mouse cornea micropocket angiogenesis assay was conducted as previously described (25). Micropellets were made of sucrose aluminum sulfate and Hydron pellets (polyhydroxyethylmethacrylate), containing VEGF (100 ng) with or without nitidine chloride (15 μg/pellet). Seven days later, eyes were photographed. Maximal vessel length and clock hours of circumferential neovascularization were measured by the Image-Pro plus 6.0 software package (Media Cybernetics Inc.) and vessel area was calculated according to the formula of 0.2π × length × clock number. Two independent experiments were carried out.

Live/dead staining assay
Apoptosis of cells was also determined by live/dead reagent (Invitrogen), which was used to measure intracellular esterase activity and plasma membrane integrity (26).
Annexin V/propidium iodide staining assay
Nitidine chloride–mediated cell apoptosis was assayed by Annexin V–fluorescein isothiocyanate (FITC) and propidium iodide staining (ApopNexin Annexin V FITC apoptosis kit; Millipore) as described earlier (23).

Immunofluorescence assay
Briefly, SGC-7901 (6 × 10⁴ cells) were treated with nitidine chloride (30 μmol/L) for phosphorylation of STAT3 (Tyr705) detection in the nuclei using immunofluorescence analysis (27). The images were captured by confocal laser scanning microscopy (LSM 5 PASCAL; Carl Zeiss; magnification, ×600).

Western blot analysis
HUVECs were first starved in serum-free ECM for 4 to 6 hours and then pretreated with nitidine chloride, followed by the stimulation with 50 ng/mL of VEGF for 2 to 20 minutes. Tumor cells were directly exposed to nitidine chloride. The whole-cell extracts were prepared by lysis buffer supplement with different kinds of protein inhibitors. Equal protein aliquot of each lysate was subjected to SDS-PAGE (6%–12%), blotted onto polyvinylidene difluoride (PVDF) membrane (Bio-Rad), probed with specific antibodies and subsequently detected by chemiluminescence. Protein concentration was determined by Micro BCA Protein Assay Kit (Pierce Biotechnology).

RNA isolation and reverse transcriptase PCR
Total RNA from SGC-7901 cancer cells treated with nitidine chloride (30 μmol/L) for different intervals was extracted with TRIzol reagent and converted to cDNA by reverse transcriptase PCR (RT-PCR) kit (Invitrogen). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control.

Electrophoretic mobility shift assay
DNA-binding activity of STAT3 was examined by electrophoretic mobility shift assay using IRDye700 infrared dye labeled oligonucleotide probe (LI-COR, Biosciences) and analyzed in both HUVECs and SGC-7901 cells based on conditions defined previously (28).

Chromatin immunoprecipitation assay
SGC-7901 cells were treated with various concentrations (10, 20, and 40 μmol/L) of nitidine chloride for 48 hours, fixed with 1% formaldehyde, and lysed as described previously (29). Chromatin samples were immunoprecipitated with antibodies against STAT3 or with normal rabbit IgG antibody and examined by quantitative PCR using the SYBR Premix Ex Taq Kit (TaKaRa Biotechnology).

Human gastric tumor xenograft mouse model
Briefly, SGC-7901 cancer cells (4 × 10⁶ cells/mouse) were injected subcutaneously into the right flank of 6-week-old male BALB/cA nude mice (National Rodent Laboratory Animal Resources, Shanghai, China). After tumor grew to about 70 mm³, the mice were randomly assigned into 2 groups (n = 8) and treated with or without nitidine chloride (7 mg/kg/d) via intraperitoneal injection for consecutive 20 days. The mice of control group were administrated with same amount of DMSO. At the same time, solid tumor volume was determined with vernier caliper measurements and the formula of A × B² × 0.52, where A is the longest diameter of the tumor and B is the shortest diameter of the tumor. After 20 days, solid tumors were harvested and analyzed for immunohistochemistry (Lifespan Biosciences) and Western blotting.

Histology and immunohistochemistry
Solid tumors were removed, fixed with 10% formaldehyde, and embedded in paraffin. Immunohistochemical staining for STAT3, VEGF, and CD31 were carried out. Images were taken with a Leica DM 4000B photo microscope (magnification, ×400).

Statistical analysis
Statistical comparisons between groups were conducted using one-way ANOVA followed by the Dunnet test. Data were presented as means ± SDs. P values of 0.05 or less were considered statistically significant.

Results
Nitidine chloride inhibits VEGF-induced endothelial cell proliferation, migration, and tubular-structure formation in vitro
To systematically address the inhibitory activity of nitidine chloride on tumor angiogenesis, we first evaluated its antiangiogenic function in vitro. As shown in Fig. 1B, nitidine chloride dose-dependently decreased VEGF-induced cell viability in HUVECs, with the IC₅₀ value of 5 μmol/L. Given the importance of vascular endothelial cell motility in the process of angiogenesis (30), we further evaluated the potential effect of nitidine chloride on endothelial cell migration. Results showed that treatment with nitidine chloride (5–10 μmol/L) remarkably impaired the chemotactic motility of HUVECs in Boyden chamber assays (Fig. 1C). The invasive cell number of nitidine chloride–treated HUVECs was much less than that of the control group (P < 0.01).

To better characterize the inhibitory function of nitidine chloride on neovascularization, we conducted 2-dimensional Matrigel assays. As shown in Fig. 1D, endothelial cells differentiate and spontaneously align to form a capillary structure network on Matrigel layer (Control). Nitidine chloride at tested concentrations (>1 μmol/L) significantly suppressed or inhibited the tubular structure formation of endothelial cells (Fig. 1D).

Nitidine chloride inhibits VEGF-induced angiogenesis in vivo
We further explored the antiangiogenic activity of nitidine chloride using 2 in vivo angiogenesis models,
the Matrigel plug assay, and the mouse corneal micropocket assay. As showed in Fig. 2A, Matrigel plugs containing VEGF alone appeared dark red, indicating that functional vasculatures had formed inside the Matrigel via angiogenesis triggered by VEGF. In contrast, the addition of different amounts of nitidine chloride (30 or 60 µg per plug) to the Matrigel plugs containing VEGF dramatically inhibited vascular formation (Fig. 2A). These plugs displayed much paler appearance at 30 µg and showed almost no vascular formation at 60 µg (Fig. 2A, bottom, VEGF + NC 60 µg). Immunohistochemistry staining indicates that large amount of CD31-positive endothelial cells existed inside the VEGF alone plugs, whereas the number of CD31-positive endothelial cells in nitidine chloride-treated plugs had dramatically decreased (Fig. 2B).

We next confirmed the in vivo antiangiogenic functions of nitidine chloride in the mouse corneal micropocket assay. As shown in Fig. 2C, VEGF (100 ng) could significantly induce neovascularization in mouse cornea. In contrast, treatment with nitidine chloride (15 µg/mouse) led to a predominant reduction of angiogenesis in corneal vessel area as indicated by vessel length, clock number, and vessel area. These results indicated that nitidine chloride inhibited VEGF-induced angiogenesis in vivo.

**Nitidine chloride blocks VEGF-induced STAT3 activation in endothelial cells**

To figure out the molecular basis of nitidine chloride in antiangiogenesis, we examined the signaling pathways mediated by nitidine chloride in HUVECs using Western blot analysis and electrophoretic mobility shift assay (EMSA). It has been known that VEGF signaling events relevant to tumor angiogenesis are mainly mediated by VEGF receptor 2 (VEGFR2) phosphorylation (23). Therefore, we first tested the action of nitidine chloride on this critical receptor tyrosine kinase on endothelial cell membrane. We found that 5 µmol/L of nitidine chloride significantly inhibited the phosphorylation of VEGFR2 at Tyr1,175 site (Fig. 3A). Because VEGF can trigger the activation of STAT3 signaling in HUVECs, we examined the effects of nitidine chloride on the phosphorylation of STAT3 and showed that nitidine chloride dose- [Fig. 3B (i)] and time [Fig. 3B (ii)] dependently suppressed the phosphorylation of STAT3 (Tyr1,055), with the maximum inhibition at 5 to 10 µmol/L. When noted, such inhibitory

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**Figure 2.** Nitidine chloride inhibits VEGF-induced angiogenesis in vivo. A, representative images of Matrigel plugs in each group (n = 4–6). B, immunohistochemistry analysis with CD31 antibody was conducted on the sections of Matrigel plugs (magnification, ×400), showing CD31-positive endothelial cells. C, representative images of neovascularization in mouse corneal assay. Columns, mean from 2 independent experiments; bars, SD; **, P < 0.01 versus VEGF alone group.
action of nitidine chloride on STAT3 was in parallel with a rapid dephosphorylation of upstream kinases of STAT3, including Janus activated kinase 1 (JAK1; Tyr1,022/1,023), JAK2 (Tyr1,007/1,008), and c-Src (Tyr416; Fig. 3B).

STAT3 translocation to the nucleus results in a specific DNA binding that in turn regulates target gene transcription, we next determined whether nitidine chloride suppressed DNA-binding activity of STAT3 by EMSA assay. Our results showed that nitidine chloride decreased STAT3 DNA-binding activity in a concentration-dependent manner (Fig. 3C). These results provide evidence that nitidine chloride blocked angiogenesis by targeting STAT3 signaling pathway.

Nitidine chloride suppresses STAT3 signaling in gastric cancer cells

Our study showed that nitidine chloride exerts anti-angiogenic activity in vitro and in vivo through blocking STAT3 pathway in endothelial cells, with effective concentrations around 10 μmol/L, suggesting that STAT3 is a potential target of nitidine chloride in gastric cancer cells. To address such a possibility, we examined the inhibitory effect of nitidine chloride on STAT3 in 2 human gastric cancer cell lines, SGC-7901 and AGS, which are known to express constitutively active STAT3 protein (31, 32). As expected, nitidine chloride blocked STAT3 phosphorylation in SGC-7901 cancer cells in dose- and time-dependent manners in both whole-cell lysate (Fig. 4A) and the nuclear translocation assays (Supplementary Fig. 5B). We similarly verified the effects of nitidine chloride on activity of STAT3 in AGS gastric cancer cells (Supplementary Fig. 5A). Moreover, DNA-binding ability of STAT3 was also remarkably inhibited by nitidine chloride, as indicated by EMSA analysis (Fig. 4B), with an effective concentration of 20 μmol/L.

Nitidine chloride suppresses STAT3 transcriptional activity in gastric cancer cells

STAT3 participates in oncogenesis through upregulation of genes encoding apoptosis inhibitors (Bcl-xL, Bcl-2, and survivin), cell-cycle regulators (cyclin D1 and c-Myc), and inducers of angiogenesis (VEGF; ref. 8). We next examined whether the expression of these STAT3 target genes is downregulated by nitidine chloride in SGC-7901 cancer cells. Our results showed that both mRNA and protein levels of cyclin D1, Bcl-2, Bcl-xL, and VEGF were significantly decreased by the treatment of nitidine chloride, with maximum suppression observed at around 12 to 24 hours (Fig. 4C). In agreement, in vivo binding of Bcl-xL, cyclin D1, and VEGF to STAT3 was also dose-dependently inhibited by nitidine chloride analyzed by chromatin immunoprecipitation (Fig. 4D).
Nitidine chloride inhibits cell viability and induces apoptosis in gastric cancer cells

Because nitidine chloride suppressed the activation of STAT3 and STAT3-regulated proliferative gene products, we speculated that this compound could block cancer cell growth. Results in Fig. 5A showed that nitidine chloride remarkably suppressed the viability of several gastric cancer cell lines, with an IC$_{50}$ value of about 20 µmol/L. In addition, we further investigated the proapoptotic effects of nitidine chloride by live-dead staining, flow cytometry, and Western blot assays. As shown in Fig. 5B, nitidine chloride dose-dependently potentiated cancer cell death, with an IC$_{50}$ value at about 20 µmol/L. Similarly, the proportion of apoptotic cells was significantly increased from 7.27% to 39.36% after 48-hour treatment (Fig. 5C), and clear cleavages of PARP and caspase-3 occurred when treated with 20 µmol/L of nitidine chloride (Fig. 5D).

Nitidine chloride suppresses tumor angiogenesis and tumor growth in a human gastric cancer xenograft mouse model

To evaluate the efficacy of nitidine chloride on tumor growth and tumor angiogenesis in vivo, we further transplanted SGC-7901 cells into mice and constructed a human gastric cancer xenograft mouse model. We found that intraperitoneal administration of nitidine chloride (7 mg/kg/d, 20 days) led to significant inhibition of tumor volume [Fig. 6A (i)] and tumor weight [Fig. 6A (ii), $P < 0.001$], without noticeable toxicity [Fig. 6A (iii)] as compared with the counterparts treated with DMSO. The average volume of solid tumors in nitidine chloride–treated mice (300.51 $\pm$ 53.13 mm$^3$) was much smaller than that of control group ($796.84 \pm 79.21$ mm$^3$; Fig. 6B (i)). Interestingly, when the skin of each mouse was pulled back to expose an intact tumor, we found that tumor growth suppression mediated by
Nitidine chloride was well correlated with angiogenesis inhibition [Fig. 6B (ii)].

To examine the molecular target and neovascularization in solid tumors, we further carried out immunohistochemistry with STAT3, CD31, and VEGF antibodies. As shown by Fig. 6C, nitidine chloride dramatically reduced the expression of STAT3 and VEGF in solid tumors. The number of CD31-positive endothelial cells in the nitidine chloride–treated xenografts accordingly decreased. Both the levels of STAT3 phosphorylation and total STAT3 protein in randomly selected tumors of the 2 groups were also remarkably different, as showed by Western blotting results (Fig. 6D). Together, our findings showed that nitidine chloride significantly suppressed both tumor growth and tumor angiogenesis in vivo by interfering with STAT3 signaling pathway.

Discussion

More recently, increasing attention has been devoted to the aberrant activation of STAT3 for its critical role in tumor progression (33–35). However, therapeutic agents targeting STAT3 signaling pathway are still limited. In our present study, we clearly evaluated the anticancer potential of nitidine chloride in vitro and in vivo, and found, for the first time, that nitidine chloride effectively inhibited tumor angiogenesis and tumor growth of human gastric cancer by interfering with STAT3 signaling pathway.

Angiogenesis has been an attractive target for drug therapy due to its pivotal role in tumor growth and metastasis (4, 36), and antiangiogenic therapy is now considered as the forth strategy to treat cancer (4, 37). Angiogenesis is a complex multistep process that involves endothelial cell proliferation, migration, and tube formation triggered by specific growth factors in tumor microenvironment. In this study, we found that nitidine chloride effectively abrogated VEGF-induced HUVECs proliferation, invasion, and capillary-like structures formation in vitro, even at sublethal concentrations. In addition, VEGF-triggered neovascularization in the Matrigel plugs and in the mouse cornea in vivo were also dramatically suppressed by nitidine chloride, providing a critical clue to the ability of this alkaloid to inhibit angiogenesis. When compared the effective concentrations of nitidine chloride on endothelial cells (Fig. 1, 5–10 μmol/L) and gastric epithelial cells (Fig. 5, 20–30 μmol/L), we found that antiangiogenic activity of nitidine chloride may be much earlier than its cytotoxic effects on tumor cells.

Previous reports have shown a close association between STAT3 activation and gastric cancer growth and
revascularization (38, 39). Moreover, activation of STAT3 has been directly correlated with VEGF production (40). Our present investigation showed that both mRNA and protein expression of VEGF were dose-dependently suppressed by nitidine chloride via STAT3 inhibition in human gastric cancer cells. VEGF was initially thought to mediate its signaling in a paracrine manner by acting on neighboring endothelial cells via VEGFR2, which was specifically expressed on the endothelial cell surface and believed to regulate the majority of the angiogenic effects of VEGF (23, 36, 41). In this study, we found that nitidine chloride dose-dependently inhibited the phosphorylation of VEGFR2 (Tyr1175) in HUVECs, which may lead to the inhibition of a number of downstream signaling cascades. STAT3 is principally activated by nonreceptor tyrosine kinase JAK2 (42), and c-Src family kinase has also been involved in STAT3 phosphorylation (43). Our results showed that phosphorylation of Src (Tyr416), JAK1 (Tyr1022/1023), and JAK2 (Tyr1007/1008) was dose- and time-dependently blocked by nitidine chloride in endothelial cells (Fig. 3), indicating that the direct effects of nitidine chloride on angiogenesis might be through inhibiting the VEGFR2/STAT3 signaling cascade. As evidenced by our previous reports, other natural compounds, such as indirubin (44) and cucurbitacin E (45), similarly inhibited tumor angiogenesis via blocking VEGFR2-mediated JAK2/STAT3 pathway.

As previously mentioned, STAT3 participates in tumorigenesis through upregulation of genes encoding apoptosis inhibitors (Bcl-xL, Bcl-2, and survivin) and cell-cycle regulators (cyclin D1 and c-Myc; ref. 46). In this study, we found that nitidine chloride blocked the activation STAT3 in SGC-7901 and AGS gastric cancer cells, indicating that the STAT3 suppressive activity of nitidine is not cell type specific. Bcl-xL is an antiapoptotic protein of the Bcl-2 family that inhibits apoptosis by preventing cytochrome c release (47). High level of Bcl-xL expression has been associated with advanced malignancies and poor
prognosis (48). Therefore, suppression of Bcl-2 and Bcl-xL expression may be a useful strategy to potentiate cancer apoptosis (49, 50). Notably, we found that STAT3-dependent target genes including Bcl-2 and Bcl-xL were dose-dependently downregulated by nitidine chloride. Constitutively active STAT3 has been implicated in the induction of resistance to apoptosis, possibly through the expression of Bcl-2, Bcl-xL, and cyclin D1 (46). Our findings implied that suppression of STAT3 activation by nitidine chloride could facilitate cancer cell apoptosis. In agreement with this assumption, nitidine-treated SGC-7901 gastric cancer cells undergo clear death and apoptosis, as indicated by increased cleavage of caspase-3 and PARP in cytoplasm (Fig. 5). In addition, proliferative (cyclin D1), antiapoptotic (Bcl-2 and Bcl-xL), and proangiogenic genes (VEGF) of STAT3 were all downregulated by the treatment of nitidine, suggesting a positive-feedback loop and a close association between nitidine chloride–mediated inhibition on angiogenesis and tumor growth. However, further studies are needed to carefully substantiate this speculation.

To evaluate the antitumor activity of nitidine chloride in vivo, athymic nude mice were transplanted with human gastric cancer cells and treated with nitidine chloride. We found that nitidine chloride significantly suppressed the growth of gastric tumor xenografts, the inhibition rate of 65% at dosage of 7 mg/kg/d) and also dramatically reduced neovascularization. Considering the effective antiangiogenesis of nitidine chloride in the Matrigel plugs and mouse cornea, we deem that nitidine chloride inhibits tumor growth in vivo, not only directly inhibiting tumor cell proliferation but also through suppression of tumor angiogenesis. We further clearly showed a reduced expression of VEGF and STAT3 protein in the tumors of nitidine-treated mice as compared with the tumors of DMSO-treated mice. Taken together, our findings is the first to provide convincing evidence that nitidine chloride suppresses gastric tumor growth and tumor angiogenesis by suppressing constitutively activated STAT3 signaling cascade, making the compound a promising agent for the treatment of cancer and angiogenesis-related pathologies. However, further studies are needed to carefully substantiate this speculation on the precise target of nitidine chloride in coordinately suppression of angiogenesis and tumor growth.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

This work is supported by the grants from National Natural Science Foundation of China (81101683 and 3090055), Chenguang Program from Shanghai Municipal Education Commission (10CG25), a grant from NIH (R01 CA134731), and Fundamental Research Funds for the Central Universities (78210021).

Received August 21, 2011; revised December 2, 2011; accepted December 7, 2011; published OnlineFirst December 27, 2011.

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286 Mol Cancer Ther; 11(2) February 2012

Molecular Cancer Therapeutics

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