Original Articles

Inhibition of the NF-κB pathway by nafamostat mesilate suppresses colorectal cancer growth and metastasis

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ABSTRACT

Nafamostat mesilate is an anti-inflammatory drug that is usually used to treat pancreatitis. Recent studies show that it can suppress pancreatic cancer via inhibition of the nuclear factor κB (NF-κB) pathway. However, whether it has anti-tumor activity in some other cancer, including colorectal cancer (CRC), has not been investigated and remained unclear. Here, our study showed that nafamostat mesilate abrogated the constitutive NF-κB activation in CRC cells, which is mediated through phosphorylation of IκBα and nuclear translocation of p65. Also, we found that nafamostat mesilate inhibited phosphorylation of Erk in CRC cells. Consistently, our study demonstrated that nafamostat mesilate inhibited the CRC cell proliferation, invasion and migration and induced mitochondria-dependent apoptosis. Furthermore, nafamostat mesilate could reverse oxaliplatin induced NF-κB and Erk activation in CRC cells, and enhance the sensitivity of CRC cells to oxaliplatin. Nafamostat mesilate combined with oxaliplatin repressed subcutaneous tumor growth and hepatic metastasis in vivo. Overall, our data suggest that nafamostat mesilate, a relatively non-toxic drug that targets NF-κB and Erk, may, in combination with oxaliplatin, represent a novel therapeutic strategy for CRC treatment.

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Introduction

Colorectal cancer (CRC) is the leading cause of cancer-related death worldwide [1,2]. Recent studies have identified the characteristic of earlier onset age in CRC patients [3]. Usually, surgical resection provides curative possibility for early stage patients, while for advanced CRC, oxaliplatin plus 5-fluorouracil and leucovorin (FOLFIRI) are recommended [4]. However, nearly all patients with metastatic lesions develop chemoresistance to oxaliplatin with a median progression time of approximate 8 months [5]. Therefore, it is urgent to develop effective strategies to enhance the sensitivity of CRC to oxaliplatin.

Many factors related to oxaliplatin resistance have been identified by our group and others. These include low expression of the circadian clock gene, Bmal1 [6], epithelial-to-mesenchymal transition [7] and activation of specific pathways including NF-κB [8,9] and Erk [10]. NF-κB is a typical heterodimer transcription factor consisting of p50 and p65 and plays central roles in immunity, inflammation, angiogenesis, cell proliferation, differentiation and apoptosis. Inactivated NF-κB/p65 resides in the cytosol and forms a complex with the inhibitory protein IκBα. Following stimulation by a variety of extracellular signals, the enzyme IκB kinase (IKK) is activated and phosphorylates the IκBα protein. Phosphorylation of IκBα results in ubiquitination, dissociation of IκBα from NF-κB/p65, and eventual degradation of IκBα by the proteosome. The activated NF-κB/p65 is then translocated into the nucleus where it initiates target gene transcription [11]. Constitutively activated NF-κB/p65 contributes to the aggressive behaviors of CRC [12] and other malignancies [8,13].

Thus disruption of NF-κB signaling by non-coding RNAs [13,14] or inhibitors [15] could interrupt the infinite proliferation and distant metastasis of malignant transformed cells. Nafamostat mesilate (Fut) is an anti-inflammatory drug approved for pancreatitis that inhibits enterokinase and trypsin activity [16]. Moreover, nafamostat mesilate has recently been shown to be a potent inhibitor of NF-κB and Erk pathways and could suppress pancreatic cancer with or without gemcitabine [8,17]. In combination with paclitaxel,
nafamostat mesilate inhibited the peritoneal dissemination of gastric cancer [18]. However, the effects of nafamostat mesilate on CRC and tumor metastasis and its underlying molecular mechanisms have not yet been investigated.

Here, we investigated the antitumor activity and the underlying mechanisms of nafamostat mesilate in CRC cells in vitro and in vivo. We found that nafamostat mesilate significantly suppressed proliferation, migration and invasion and induced apoptosis of CRC cells. Moreover, nafamostat mesilate enhanced the sensitivity of CRC cells to oxaliplatin and suppressed tumor growth and liver metastasis in mouse models. These results suggest that targeting the NF-κB and Erk pathways by nafamostat mesilate, a relatively non-toxic drug approved for pancreatitis, may, in combination with oxaliplatin, represent a novel therapeutic strategy for CRC treatment.

Materials and methods

Cell lines and cell culture

The human CRC cell lines Colo 205, CV-2, DLD-1, LS174T, RKO, SW 480, HCT 116, SW 1116, SW 620, HT-29 and LoVo and the colon epithelial cell CDD 112 were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured and according to the instructions. All cells were cultured in RPMI medium supplemented with 10% FBS (ThermoFisher Scientific, Carlsbad, California, USA) at 37 °C with 5% CO2. All cell lines were authenticated by short tandem repeat DNA fingerprinting and tested for mycoplasma at Medicine Lab of Forensic Medicine Department of Sun Yat-sen University (Guangzhou, China) before use.

Reagents and antibodies

Nafamostat mesilate, oxaliplatin and U0126 were purchased from Selleck Chemicals (Houston, TX, USA) and dissolved in DMSO. Antibodies against p65, phospho-(p)-IkBα, IkBα, p-IkBα, p-IκBα, IκBα, β-Actin, cleaved (c)-PARP, c-Caspase3, c-Caspase5, p-ERK, Akt, p-Akt, Erk, GAPDH (Cell Signaling Technology, Beverly, MA, USA), Ki-67 and Lamin B1 (Abcam, Cambridge, Massachusetts, USA) were used for immunoblotting.

qRT-PCR

Total RNA was obtained using TRIzol regent (Life Technologies, Carlsbad, California, USA) and reverse transcribed to cDNA using a Takara kit (HRK, Japan). The mRNA expression levels were measured by qRT-PCR using a LightCycler 480 instrument (Roche Diagnostics, Basel, Switzerland) as described in a previous report [19]. All samples were analyzed in triplicate in a 10 μl volume. Specificity was verified by melting curve analysis, and the expression of target genes was normalized to that of GAPDH. Data were analyzed using the 2−∆∆Ct method. Primers were synthesized by Life Technologies, and they are listed in Supplementary Table S1.

Western blot and immunofluorescence analysis

Proteins were extracted with radioimmunoprecipitation assay (RIPA) and quantified using a BCA assay as previously described [6]. Briefly, the medium of the indicated cells was discarded and the cells were washed twice with cold PBS. The cells were scraped off the plate, pelleted, and resuspended in the RIPA buffer. After the samples were lysed on ice for 15 min, they were centrifuged, and the supernatant was collected. A BCA kit was used to quantify each sample, and the samples were separated using 8–15% SD–PAGE and transferred to the polyvinylidene fluoride membranes (Immobilon-P, Millipore, Bedford, USA). The membranes were then blocked with 5% nonfat milk in TBST for 1 h at room temperature before they were incubated with the indicated primary antibodies diluted in 5% bovine serum albumin in TBST at 4 °C overnight. After three washes with TBST, the membranes were probed with peroxidase-linked secondary antibody for 1 h at room temperature. Enhanced chemiluminescence (Supersignal ECL, ThermoFisher Scientific, Carlsbad, USA) was used to visualize the proteins in the membrane. Nuclear and cytoplasmic extracts were obtained with a nuclear protein kit (KeyGEN, Nanjing, China) according to the manufacturer’s instructions. Immunofluorescence of p65 was detected using a standard protocol [20].

Cell apoptosis and proliferation assays

Cell apoptosis and the decrease of mitochondrial membrane potential induced by nafamostat mesilate or oxaliplatin were determined by AnnexinV/PI (KeyGEN, Nanjing, China) and JC-1 (Beyotime, Shanghai, China) staining, respectively, followed by flow cytometer analysis (Beckman Coulter, California, USA) according to the manufacturer’s instructions. CCK-8 (Dojindo, Japan) and colonic assays were used to detect cell viability and proliferation, as described previously [20].

siRNA transfection and dual-luciferase reporter assay

Specific siRNAs targeting p65 were purchased from Ribobio (Guangzhou, China). Transfection of the siRNAs was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) according to the manufacturer’s instructions as described previously [21]. Briefly, growing cells seeded at 4×105 cell per well in a 6-well plate were transfected with 10 nmol siRNA. After 48 h, the cells were trypsinized for transwell assay, or proteins were extracted to validate the transfection efficiency. For the dual-luciferase reporter assay, HCT 116 or SW 1116 cells were transfected with the NF-κB/p65 luciferase reporter and pRL-TK Renilla luciferase construct (Promega) per well using Lipofectamine 2000. After 24 h, the cells were treated with the indicated chemicals. The cells were then analyzed after an additional 24 h according to the Dual Luciferase Assay System protocol (Promega).

Transwell migration and invasion assays

Transwell chambers (Corning, New York, USA) were used to evaluate the migration and invasion of cells treated with nafamostat mesilate or oxaliplatin as previously reported [19]. Briefly, cells pretreated with the indicated chemicals were trypsinized and resuspended with medium lacking FBS. For the migration assay, 1×105 cells suspended in 200 μl of medium were added to the upper chamber, and 600 μl of medium with 50% FBS was added to the lower chamber as a chemoattractant. After a 24 h incubation, cells that migrated to the lower chamber were fixed with methanol, stained by crystal violet (Sigma-Aldrich, St. Louis, USA), dried at room temperature and imaged using a microscope, while cells remaining in the upper were wiped off with cotton swabs. For the invasion assay, 2×105 cells were added to the upper chamber of the inserts with matrix gel, and 600 μl of medium with 50% FBS was added to the lower chamber. The invading cells were stained by crystal violet and imaged after 30 h of culturing. Microscopic counts of the migrated or invaded cells were determined in five different fields.

Animal study

Female BABL/c nude mice (4–5 weeks old) were purchased from the Guangdong Province Laboratory Animal Center (Guangzhou, China). To evaluate the antitumor effects of nafamostat mesilate with or without oxaliplatin, two experimental animal models were used. First, HCT 116 cells (1×106) were subcutaneously injected to the right armpit of the mice. After one week, the mice were assigned to the following groups: control, PBS; nafamostat mesilate, 30 mg/kg, thrice per week; oxaliplatin, 10 mg/kg, once per week; combination, oxaliplatin, 10 mg/kg, once per week followed by nafamostat mesilate, 30 mg/kg, thrice per week. Tumor volumes were examined twice per week. After four weeks of treatment, the mice were sacrificed, and the tumors were removed, embedded in paraffin and sectioned. Second, to determine whether CRC metastasis could be prevented by nafamostat mesilate with or without oxaliplatin, the following previously described methods [19] were used. Briefly, after the mice were anesthetized by inhalation of isoflurane (0.5–1.0%) and oxygen, the spleen was located via a 1 cm incision in the upper left lateral abdomen. HCT 116 cells (1×106) suspended in 20 μl of PBS were injected into the distal tip of the spleen using a Hamilton syringe. The spleen was then gently replaced, and the incision was closed. One week after the operation, the animals were injected with different chemicals for 4 weeks. The mice were then sacrificed, and the spleen and liver were collected, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) to detect micrometastasis. Our animal study was approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University.

Histological and immunohistochemical analyses

Tumor tissues from the BCLB/c nude mice were stained with H&E or were immunohistochemically stained for Ki-67 using previously reported protocols [19]. TdT-mediated dUTP nick and labeling (TUNEL) staining using cell death detection kit (Biotool, Houston, TX, USA) was also used to identify apoptotic cells in situ according to the manufacturer’s instructions. Briefly, formalin-fixed paraffin-embedded mouse tumor tissue were sectioned, dehydrated in xylene, hydrated in an ethanol gradient, digested using proteinase K, and incubated with specific probes. The nuclei were counterstained using DAPI (Invitrogen, Carlsbad, USA) before they were mounted with ProLong Gold antifade reagent (Invitrogen, Carlsbad, USA). For each sample, three random fields were selected to count the total number of TUNEL-positive cells, and representative images were taken using an Olympus FX1000 microscope (Olympus, Tokyo, Japan).

Statistical analysis

Data are presented as mean ± SD. For comparison of the statistical differences among more than two groups, one-way ANOVA and Newman–Keuls multiple comparison tests were used, while other comparisons were performed with the Student’s unpaired t-test using the GraphPad Prism software (San Diego, CA, USA). P<0.05 was considered significant.
Results

Nafamostat mesilate disrupts NF-κB signaling via suppression of p65 nuclear translocation and IκBα phosphorylation in vitro

Because persistent activation of the NF-κB pathway has been shown in several human cancers including CRC [22], we investigated NF-κB/p65 expression in a panel of CRC cells and one immortalized colon epithelial cell line, CCD 112. Nuclear p65, the active form that binds DNA to initiate gene transcription, was overexpressed in CRC cell lines compared with that in CCD 112 cells (Fig. 1A). Inhibition of NF-κB/p65 activity by nafamostat mesilate (Fig. 1B) has been reported in pancreatic cancer [17]. To determine whether nafamostat mesilate could also abrogate NF-κB/p65 activity in CRC, we examined the changes in subcellular localization and expression of nuclear NF-κB/p65 after treatment with 100 μM nafamostat mesilate for 24 h. The cytokine TNFα, which is known to activate NF-κB/p65, was used as a positive control. Nafamostat mesilate treatment caused a substantial decrease in p65 localized in the nucleus induced by TNFα in HCT 116 and SW 1116 cells (Fig. 1C). Furthermore, we also found that nuclear NF-κB/p65 expression was induced by TNFα, and this effect was reversed by transfection with specific siRNAs targeting NF-κB/p65 or co-treatment with nafamostat mesilate in HCT 116 and SW 1116 cells (Fig. 1D) as determined by analysis of nuclear protein expression. Accordingly, the expression levels of several known NF-κB target genes related to cell proliferation and apoptosis were significantly decreased in CRC cells treated with 100 μM nafamostat mesilate for 24 h (Fig. 1E). To explore the mechanism underlying the suppressive role of nafamostat mesilate on NF-κB, we evaluated the expression levels of IκBα, the physiological inhibitor of NF-κB. Fig. 1F shows that nafamostat mesilate suppressed the phosphorylation of IκBα in HCT 116, SW 1116 and SW 620 cells in a dose-dependent manner. Further analysis indicated that nafamostat mesilate suppressed the phosphorylation of Erk (Fig. 1G) while Akt remained unchanged (Supplementary Fig. S1), which was consistent with a previous report [23]. Taken together, our data suggest that nafamostat mesilate inhibited NF-κB activation through the suppression of p65 nuclear translocation and IκBα phosphorylation in CRC cell lines.

Nafamostat mesilate inhibits proliferation, migration and invasion of CRC cells

To test our hypothesis that pharmacological inhibition of NF-κB/p65 by nafamostat mesilate modulates the malignant properties of CRC, we used CCK-8 assays and demonstrated that cell viability was significantly decreased by nafamostat mesilate treatment in a time and dose-dependent manner (Fig. 2A and B). In contrast, nafamostat mesilate was less cytotoxic to the CCD 112 colon epithelial cells, suggesting that nafamostat mesilate could selectively kill CRC cells. In addition, colony formation assays demonstrated approximately 60% and 90% decrease in colony numbers after treatment with 50 μM nafamostat mesilate in HCT 116 and SW 1116 cells, respectively (Fig. 2C). Further analysis showed that nafamostat mesilate inhibited the migration (Fig. 2D) and invasion (Fig. 2E) of both CRC cell lines in a time dependent manner. Finally, we observed decreased colony numbers and inhibition of migration and invasion after nafamostat mesilate treatment in SW 620 cells (Supplementary Fig. S2A and B). However, siRNA mediated depletion of NF-κB/p65 expression attenuated the anti-migration activity of nafamostat mesilate in HCT 116 and SW 1116 cells (Fig. 2F and G), further demonstrating that nafamostat mesilate suppressed the migration of CRC cells via the NF-κB pathway. Nafamostat mesilate could not cause further decrease of cell viability after pretreatment with the Erk inhibitor, U0126, in HCT 116 and SW 1116 cells, demonstrating that Erk was involved in the suppressive role of nafamostat mesilate in CRC cells. Thus, nafamostat mesilate specifically inhibits proliferation, migration and invasion of CRC cells via inhibition of NF-κB/p65 and p-Erk in vitro.

Nafamostat mesilate induces cell apoptosis in CRC

To determine whether nafamostat mesilate has a pro-apoptotic effect on CRC cells, we used flow cytometry analysis. As shown in Fig. 3A, the mitochondrial membrane potential, which is affected prior to membrane erosion in cells undergoing apoptosis and is detected with JC-1 staining when impaired, was significantly decreased following nafamostat mesilate treatment. Moreover, Annexin-V/PI dual staining in both HCT 116 and SW 1116 cells revealed that nafamostat mesilate caused a time-dependent increase in apoptotic cells (Fig. 3B and C). The pro-apoptotic effects were further validated in SW 620 and LoVo cells (Supplementary Fig. S3A and B). Caspase9, caspase3 and PARP cleavage, markers of apoptosis, were upregulated in HCT 116 and SW 1116 cells after nafamostat mesilate treatment (Fig. 3D). In sum, these results indicated that nafamostat mesilate can induce apoptosis in CRC cells.

Nafamostat mesilate enhances sensitivity to oxaliplatin in CRC cells

Oxaliplatin-based chemotherapy is an important clinical regimen used for the treatment of CRC [24]. Chemoresistance is a common cause of treatment failure. The aberrant activation of NF-κB signaling is considered one possible mechanism underlying treatment failure [25]. Intriguingly, nafamostat mesilate treatment of SW 1116 cells reversed the elevated expression of p-IκKβ/β and p-IκBα induced by oxaliplatin, but these effects were not further validated in HCT 116 cells, indicating a cell type-specific response to oxaliplatin (Fig. 4A and B). Luciferase reporter assay demonstrated that NF-κB was activated by oxaliplatin in SW 1116 cells, but not in HCT 116 cells (Fig. 4C). Concordantly, upregulation of NF-κB target genes was observed only in SW 1116 cells but not in HCT 116 cells after oxaliplatin exposure (Supplementary Fig. S4A). However, the phosphorylation of Erk was induced in both cells (Supplementary Fig. S4B and C) following oxaliplatin treatment. Upregulation of p-Erk was blocked by co-treatment with nafamostat mesilate (Fig. 4D). We therefore hypothesized that nafamostat mesilate could enhance sensitivity to oxaliplatin in CRC cells in vitro. First, oxaliplatin plus nafamostat mesilate significantly decreased the viability of HCT 116 and SW 1116 cells compared with that of oxaliplatin alone (Fig. 5A). The combination index (CI) value was less than 1, indicating synergistic effects between oxaliplatin plus nafamostat mesilate (Supplementary Fig. S4D). Second, although either agent alone caused some delay in cell growth in HCT 116 and SW 1116 cells, together nafamostat mesilate and oxaliplatin showed synergistic inhibition of colony formation (Fig. 5B). Transwell analysis showed that HCT 116 and SW 1116 cells exhibited significantly decreased migration (Fig. 5C) and invasion (Supplementary Fig. S4E) in the presence of nafamostat mesilate and oxaliplatin compared to that of either agent alone. The combination effects of nafamostat mesilate and oxaliplatin were further explored with Annexin-V/PI dual-labeling and JC-1 staining. As shown in Fig. 5D, a significantly increased percentage of apoptotic cells was observed following co-treatment with oxaliplatin and nafamostat mesilate compared to that of either agent alone. The combination effects of nafamostat mesilate and oxaliplatin were further validated with Annexin-V/PI dual staining and JC-1 staining. As shown in Fig. 5E, the combination of nafamostat mesilate and oxaliplatin increased the percentage of apoptotic cells. Altogether, these data demonstrated that nafamostat mesilate sensitized CRC cells to oxaliplatin through inhibition of oxaliplatin-induced NF-κB/p65 or Erk activation as determined by proliferation, colony formation and apoptosis assay.
Fig. 1. Nafamostat mesilate inhibits NF-κB signaling via inhibition of NF-κB/p65 nuclear translocation in CRC cells. (A) Expression of nuclear p65 in CRC cell lines and the colon epithelial cell line CCD 112. Lamin B1 was used as a nuclear loading control. (B) Chemical structure of nafamostat mesilate. (C) Immunofluorescence of NF-κB/p65 in HCT 116 and SW 1116 cells exposed to TNFα (25 ng/ml) with or without nafamostat mesilate (100 μM). Scale bars: 20 μm. (D) Immunoblotting of NF-κB/p65 in nuclear and total cell extracts treated with nafamostat mesilate (100 μM), siRNA targeting NF-κB/p65 for 24 h with or without TNFα (25 ng/ml). GAPDH and Lamin B1 were used as total fraction and nuclear markers, respectively. (E) qPCR of NF-κB response genes in HCT 116 cells (upper panel) and SW 1116 (lower panel) cells treated with nafamostat mesilate (100 μM) for 24 h. (F) Immunoblotting of p-IκBα and IκBα in HCT 116, SW 1116 and SW 620 cells after nafamostat mesilate treatment at the indicated concentrations. GAPDH was used as a loading control. (G) Immunoblotting of p-Erk and t-Erk in HCT 116, SW 1116 and SW 620 cells after nafamostat mesilate treatment at the indicated concentrations. GAPDH was used as a loading control. Data in E are presented as mean ± SD (n = 3). *P < 0.05 versus the control.
Nafamostat mesilate represses CRC growth and liver metastasis in vivo, alone or in combination with oxaliplatin

To further investigate the role of nafamostat mesilate in CRC proliferation in vivo, 1 × 10^6 HCT 116 cells were subcutaneously inoculated into nude mice. When the tumors were measurable one week later, the mice were randomly assigned into four groups that received nafamostat mesilate, oxaliplatin, a combination of both agents or vehicle control for 4 weeks, respectively. Nafamostat mesilate significantly inhibited the growth of the tumor xenografts (Fig. 6A). The tumor weight of nafamostat mesilate-treated mice was significantly less than that of the control group (Fig. 6B). Immunohistochemistry staining of the excised tumor sections showed a lower expression of Ki-67, but a higher percentage of
TUNEL-positive cells compared to those of the control group, consistent with the in vitro results (Fig. 6C). Xenografts treated with oxaliplatin showed a similar inhibition to that of nafamostat mesilate, but co-treatment with both agents resulted in remarkable suppression of tumor growth during the course of the experiment (Fig. 6A–C). In contrast, there was no significant loss in body weight in the experimental animals (Fig. 6D).

Finally, to analyze the effects of nafamostat mesilate on metastasis, HCT 116 cells (1 × 10^6/mouse) were injected into the distal tip of the mouse spleen, and the mice were randomly divided into the
The treatment began one week after the injection and lasted for 4 weeks. Importantly, although all the mice formed tumors in the spleen, 8/8 mice developed liver metastasis in the control group, while the metastasis rates in the nafamostat mesilate, oxaliplatin and combination groups were 5/8, 6/8, and 2/8, respectively (Supplementary Fig. S5A and B). Moreover, histological examination showed that the metastatic nodules were significantly decreased with the combined treatment (Supplementary Fig. S5C) without body weight loss (Supplementary Fig. S5D). Overall, our data suggest that nafamostat mesilate, alone or in combination with oxaliplatin repress CRC tumor growth and liver metastasis in vivo.

**Fig. 4.** Nafamostat mesilate inhibits the oxaliplatin-induced IκBα or Erk phosphorylation. (A) HCT 116 and SW 1116 cells were treated with nafamostat mesilate (100 μM), oxaliplatin (20 μM) or both. Expressions of p-IKKα/β, IKKα, IKKβ, p-IκBα, IκBα and NFκB/p65 was detected by western blot analysis. β-Actin was used as a loading control. (B) Quantification of p-IκBα in HCT 116 and SW 1116 cells after treatment with nafamostat mesilate (100 μM), oxaliplatin (20 μM) or both. (C) NF-κB luciferase assays of HCT 116 and SW 1116 cells after treatment with nafamostat mesilate (100 μM), oxaliplatin (20 μM) or both. (D) Immunoblotting of p-Erk and t-Erk in HCT 116 and SW 1116 cells treated with nafamostat mesilate (100 μM), oxaliplatin (25 μM) or both. GAPDH was used as a loading control. Data in B and C are presented as mean ± SD (n = 3). *P < 0.05 versus the control.
As a multifaceted transcription factor that controls the expression of several genes involved in cell proliferation, cell cycle, cell migration and apoptosis, the overexpression or aberrant activation of NF-κB driven by canonical or non-canonical stimuli [11] has been implicated in CRC [12] and other malignancies [14]. We observed elevated expression of nuclear NF-κB/p65 in a panel of CRC cells compared with that of the normal colon epithelial cell line CCD 112 (Fig. 1A). Strong NF-κB/p65 nuclear staining is observed in 40% of CRC tissues, and constitutively activated NF-κB is involved in angiogenesis and chemoresistance of CRC cells [22], suggesting that targeting NF-κB may be a potentially important new strategy for CRC therapy. However, development from the initial lead compound to the final medication requires expensive, lengthy and incremental work [26]. Alternatively, finding new use(s) for existing drugs may be an ideal method for anti-tumor agent discovery shared by scientists and clinicians.

We recently reported that inhibition of the NF-κB upstream inducer, IL-1α, using a clinically available antagonist (anakinra) suppressed pancreatic cancer in vitro and in vivo [27]. In this study, we focused on nafamostat mesilate, an anti-inflammatory drug used to treat pancreatitis. In the context of cancer, intravenous injection of nafamostat mesilate at a dose of 10 mg/kg every 7 days significantly decreased liver metastasis of colon 26 cells injected into the portal vein of CDF1 mice via inhibition of thrombin-mediated platelet aggregation and plasmin-mediated collagenase activation [28]. Fujiiwara et al. reported that nafamostat mesilate suppressed adhesion and invasion of AsPC-1 and Panc-1 cells in vitro and peritoneal dissemination of pancreatic cancer in vivo via inhibition of the NF-κB pathway [17]. Immunofluorescence and western blot analysis in our study indicated that nafamostat mesilate caused a
Fig. 6. Nafamostat mesilate suppresses CRC cell growth in vivo. (A) HCT 116 cells (1 × 10⁶/mouse) were subcutaneously injected into the right flank of nude mice before they were treated with PBS, nafamostat mesilate (30 mg/kg, thrice per week), oxaliplatin (10 mg/kg, once per week) or both (n = 8). Tumor volumes were measured on the indicated days. Data are shown as mean ± SD. (B) Excised tumor weight from the four separate groups was recorded. (C) HE staining, TUNEL and immunohistochemistry of Ki-67 from the tumor sections. Scale bar: 50 μm. Right panel: Quantification of the proliferation index and apoptotic index in the tumor sections. (D) The weight of the mice in the indicated groups was evaluated at each time point. (E) Schematic graph indicates that inhibition of the NF-κB pathway by nafamostat mesilate suppresses colorectal cancer growth and metastasis. *P < 0.05 versus corresponding control.
sequestration of NF-κB/p65 in the cytoplasm via inhibition of IκBα phosphorylation in CRC cells (Fig. 1). Furthermore, nafamostat mesilate inhibited proliferation, migration and invasion, and induced apoptosis in CRC cells. Accordingly, intraperitoneal injection of nafamostat mesilate significantly repressed tumor growth and liver metastasis when cells were inoculated subcutaneously or in the distal tail of the spleen, respectively. These results suggested that modulation of the NF-κB/p65 pathway by nafamostat mesilate attenuates the malignant phenotype in CRC cells. Further analysis indicated that decreased expression of p-Erk was involved in the anti-tumor activity of nafamostat mesilate.

The third generation platinum oxaliplatin disrupts replication and transcription through cross linking with nuclear DNA. The response rate of advanced CRC to FOLFOX has been significantly improved to 40%–50% compared with 10%–20% when 5-fluorouracil is used without oxaliplatin [29], and the overall survival is generally 2 years [30]. In adjuvant settings, FOLFOX has been routinely administered and provides curative hope for median stage patients after surgery resection. However, this treatment is far from ideal, considering that nearly all initially responding patients develop resistance 6–8 months after treatment initiation [5]. The activation of NF-κB/p65 in response to chemotherapy is well-established [25,31] and this activation, which protects tumor cells from the drug pressure, is clinically undesirable, as surviving cells may emerge as resistant [31].

Emerging evidence suggests that several conventional cancer chemotherapeutic agents that activate NF-κB/p65 lead to unfavorable clinical outcome [27,32,33], and the DNA-binding ability of NF-κB/p65 has been proposed as a major mechanism contributing to chemoresistance in CRC [9,22,34]. The naturally occurring compound thymoquinone could abrogate oxaliplatin-induced activation of NF-κB/p65 and enhance sensitivity to oxaliplatin in pancreatic cancer [32]. The anti-malaria agent quinacrine acts synergistically with oxaliplatin to induce apoptosis in CRC cells via inhibition of the NF-κB pathway [9]. Combination chemotherapy using nafamostat mesilate and oxaliplatin induces synergistic cytotoxicity in pancreatic cancer [35]. Importantly, the median survival time of patients treated with gemcitabine plus intra-arterial nafamostat mesilate was approximately two months longer than that with the gemcitabine alone without adverse effects as demonstrated in a phase I study of unresectable pancreatic cancer [36]. We therefore conceptualized that both de novo and acquired resistance to therapy could be attenuated using combination therapy based on sound rationale and hypothesized that nafamostat mesilate may be a novel chemosensitizing agent. However, activation of NF-κB was only observed in SW1116 cells after oxaliplatin treatment in our study. Failure of oxaliplatin to activate NF-κB in HCT 116 cells was consistent with a previous report [31]. However, induction of the Erk pathway by oxaliplatin was found in both HCT 116 and SW 1116 cells and was further corroborated by a microarray analysis indicating that mitogen-activated protein kinase (MAPK) signaling is significantly enriched in oxaliplatin-nonresponsive pretreatment metastatic CRC liver biopsies [10]. Intriguingly, elevated expression of p-IκBα and p-Erk induced by oxaliplatin was blocked by addition of nafamostat mesilate. It was, thus, reasonable to speculate that nafamostat mesilate may sensitize CRC cells to oxaliplatin. Cell proliferation, colony formation and transwell assays indicated that the combination of nafamostat mesilate and oxaliplatin significantly suppressed the CRC malignant phenotype compared with that of either treatment alone. Both subcutaneous and liver metastatic nude mouse models revealed profound therapeutic potential of nafamostat mesilate with oxaliplatin. To the best of our knowledge, this is the first report to demonstrate that nafamostat mesilate enhances the sensitivity to oxaliplatin in CRC cells. However, the precise mechanism by which nafamostat mesilate inhibits IκBα phosphorylation and oxaliplatin induces a cell type-specific response warrants further investigation.

The key findings of our present study provide comprehensive insights into the therapeutic application of nafamostat mesilate via inhibition of NF-κB and Erk signaling in CRC. We found that suppression of phosphorylation of IκBα and Erk by nafamostat mesilate inhibited proliferation, migration and invasion and induced apoptosis in CRC in vitro and in vivo (Fig. 6E). Furthermore, sensitization of CRC cells to oxaliplatin by nafamostat mesilate was observed. Overall, our data suggest that nafamostat mesilate, a relatively non-toxic drug that targets NF-κB and Erk, may, in combination with oxaliplatin, represent a novel therapeutic strategy for CRC treatment.

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Conflict of interest

The authors declare that they have no competing interests.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2016.06.014.

References


