# Anti-tumor effect by inhibition of NF-κB activation using nafamostat mesilate for pancreatic cancer in a mouse model

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**Abstract.** Constitutive NF-κB activation plays a key role in the aggressive behavior of pancreatic cancer. We have reported that nafamostat mesilate, a serine-protease inhibitor, inhibited NF-KB activation and induced apoptosis in human pancreatic cancer cells. The aim of this study is to evaluate the therapeutic efficacy of nafamostat mesilate against pancreatic cancer. In vitro, nafamostat mesilate inhibited NF-кВ activation of human pancreatic cancer cell line (Panc-1) by suppressing I $\kappa$ B $\alpha$  phosphorylation and induced caspase-8 mediated apoptosis. In vivo, Panc-1 was implanted into the back of nude mice. Five weeks after implantation, nafamostat mesilate was injected intraperitoneally as the treatment group (n=11) three times a week for six weeks, while the control group (n=13) received vehicle only. At the end of six-week treatment, the tumors grew up to  $12.89 \pm 4.27 \text{ mm} (\text{mean} \pm \text{SD})$ in the treatment group, and  $17.93 \pm 4.45$  mm in the control group, respectively. The tumor volume and weight of the treatment group were reduced by 43 and 61% as compared with the control group. The tumor growth was significantly inhibited in the treatment group (p<0.0001). Assays of primary tumors also indicated that nafamostat mesilate inhibited NF-KB activation by suppressing IKBa phosphorylation, resulting in caspase-8 mediated apoptosis. These results suggested that nafamostat mesilate has anti-neoplastic property against experimental pancreatic cancer.

## Introduction

Pancreatic cancer is one of the most fatal human digestive cancers with an overall 5-year survival rate of only 1-4%, because of rapid tumor growth and high potential for distant metastasis. In addition, despite developments in diagnostic

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techniques and modalities, the majority of patients with pancreatic cancer are diagnosed at the advanced stage. Therefore, only 14% of patients are amenable to resection (1). Gemcitabine is currently the standard treatment for unresectable pancreatic cancer (2), but the therapeutic benefit of gemcitabine is limited (3). Besides, many patients can not receive sufficient amount of chemotherapy, because of rapid progression, deterioration of general condition, and significant adverse effects. Therefore, new therapeutic approaches, with minimal adverse effects and tolerance even in patients with poor general condition due to advanced cancer, should be developed to further improve the outcome of unresectable pancreatic cancer.

Recent studies have demonstrated that NF-KB plays an important role in the regulation of cell apoptosis, inflammation, and oncogenesis (4-7). Inhibition of NF- $\kappa$ B is considered as one of new treatment strategies for cancer patients (8-11). In addition, constitutive activation of NF-кB has been reported to play a key role in the aggressive behavior of pancreatic cancer (12-15). We have reported that nafamostat mesilate inhibits NF-KB activation by suppressing I $\kappa$ B $\alpha$  phosphorylation and induces caspase-8 mediated apoptosis of pancreatic cancer cells (16). Also, high efficacy of nafamostat mesilate combined with gemcitabine for pancreatic cancer has been demonstrated in animal experiments (17) that applied to clinical trials (18). Nafamostat mesilate, which is a serine-protease inhibitor (19,20), is widely used for treatment of pancreatitis (21), disseminated intravascular coagulation (22), and anticoagulation in hemodialysis (23) in Japan, and has only minimal adverse effects such as hyperkalemia or hyponatremia (24-26).

To evaluate the potential of nafamostat mesilate for possible clinical application to treat unresectable pancreatic cancer, especially for patients in poor status, we explored antitumor and adverse effect of nafamostat mesilate in an experimental pancreatic cancer model.

### Materials and methods

*Reagents*. Nafamostat mesilate was a kind gift from Torii Pharmaceutical Co., Ltd., (Tokyo, Japan), was dissolved to sterile distilled water (5 mg/ml) and stored at -20°C until use.

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*Cell line*. Panc-1, a human pancreatic cancer cell line, was purchased from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Gibco-BRL, NY, USA) and penicillin/streptomycin (Gibco-BRL). The cells were cultured at  $37^{\circ}$ C with 5% CO<sub>2</sub>.

Quantitative analysis of NF-KB activity. NF-KB is typically a heterodimer that consists of the p65 (RelA) and p50 proteins. In an inactive form of NF-κB proteins are sequestered in the cytoplasm with I $\kappa$ B $\alpha$ . Following I $\kappa$ B $\alpha$  phosphorylation, NF-κB proteins are released and translocated into the nucleus, where they activate transcription of target genes. For assessment of the NF-kB activity in Panc-1 cells treated with nafamostat mesilate, concentration of NF-KB p65 in the nuclear extracts was measured both in vitro and in vivo. Nucleic extracts of both nafamostat mesilate-treated and control Panc-1 cells were prepared using a nuclear extract kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's protocol. For in vitro experiment, Panc-1 cells were incubated with nafamostat mesilate (320 µg/ml) as treatment group, or with distilled water as control group for 3 h. The nuclear extracts from both in vitro and in vivo experiments were assayed using an enzyme-linked immunosorbent assay (ELISA) kit (TransAM<sup>™</sup> NF-κB; Active Motif) to detect and quantify the NF-kB activity according to the manufacturer's instructions. Briefly, 10  $\mu$ g of nuclear extract protein was incubated for 1 h at 25°C in microwell coated with an oligonucleotide containing an NF-KB p65-binding consensus sequence. Next, the nuclear extract protein was incubated with rabbit anti-NF-kB p65 antibodies (1:1000 dilution) for 1 h at 25°C, followed by incubation with peroxidase-conjugated goat anti-rabbit IgG (1:1000 dilution) for 1 h at 25°C. The peroxidase activity was visualized by the tetramethylbenzidine reaction, and the optimal density was measured at 450 nm.

Western blot analysis. To evaluate the inhibitory effect of nafamostat mesilate on NF-kB signaling due to suppression of IkBa phosphorylation, and apoptotic effect for Panc-1 cells in vitro and in vivo, IkBa, phosphorylated IkBa, procaspase-8, and cleaved caspase-8 protein level in whole cell extracts of Panc-1 cells treated with nafamostat mesilate were determined by Western blot analysis. Protease inhibitor cocktail and phosphatase inhibitor cocktail tablets were purchased from Roche Diagnostics (Indianapolis, IN, USA). Pro- and cleaved caspase-8,  $I\kappa B\alpha$ , phosphorylated  $I\kappa B\alpha$ monoclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). For in vitro experiment, Panc-1 cells were incubated with nafamostat mesilate (320  $\mu$ g/ml) as the treatment group, or with distilled water as control group for 3 or 24 h. Protein samples of nafamostat mesilate treated and control Panc-1 cells for SDS-PAGE were prepared according to the procedure previously described (27). These samples from both in vitro and in vivo experiments were resolved by SDS-PAGE on 4-20% acrylamide gradient gels using Tris-glycine buffer and transferred onto a nitrocellulose membrane. The blotted membranes were blocked with incubation in Tris-buffered saline (TBS) containing 0.1% casein, and 0.05% Tween-20® (MP Biomedicals, Solon, OH, USA ) at room temperature for 2 h. Immunostaining was performed by incubating the blots in each primary antibody at appropriate dilution overnight. After brief washing, the membranes were incubated with the alkaline-phosphatase-labeled secondary antibody (Histofine, Nichirei, Tokyo, Japan) for 2 h and developed by using nitro blue tetrazolium/5-Bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) reagent (Bio-Rad, Hercules, CA, USA).

*Cell proliferation assay.* For evaluating the anti-tumor effect of nafamostat mesilate, the cell proliferation of Panc-1 cells after nafamostat mesilate treatment was measured. Panc-1 cells  $(1x10^4)$  seeded into a 96-well plate were incubated with nafamostat mesilate (320  $\mu$ g/ml) as treatment group, or with distilled water as control group for 24, 48 or 72 h. The cell proliferation was measured with Progma celltiter 96 Aquenous One Solution Cell Proliferation Assay (Progma, Madison, WI, USA) following the manufacturer's instructions.

Cell cycle analysis. For evaluating the induction of apoptosis by nafamostat mesilate, cell cycle analysis was performed. Panc-1 cells were incubated with nafamostat mesilate (320  $\mu$ g/ ml) as treatment group, or with distilled water as control group for 24 h. The cell cycle was analyzed by flow cytometry. In brief, the cells were harvested and  $1 \times 10^6$  were fixed with 70% (v/v) ethanol stored at -20°C until use. After centrifugation, the cell pellet was washed with phosphatebuffer saline (PBS). The cells were resuspended in PBS containing propidium iodode (50  $\mu$ g/ml) and incubated at room temperature for 10 min, followed by incubation with 0.1% Triton® X-100 (MP Biomedicals) for 10 min. The cells were incubated with 0.1% sodium citrate and DNase-free RNase (1 µg/ml) for 10 min. DNA content was determined with a FACScan flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA).

Animals and xenograft pancreatic cancer model. Five-week old male nude mice (BALBc nu/nu), were purchased from CLEA Japan Incorporated (Tokyo, Japan). The animals were housed under specific pathogen-free conditions in a biological cabinet at the Laboratory Animal Facility of Jikei University School of Medicine. The animals were maintained with a 12-h light-dark cycle at a temperature of  $22\pm2^{\circ}C$  and  $55\pm5\%$  humidity in a room with a filtered air supply.

A mouse pancreatic cancer model was established by injection of Panc-1 cells (5x106 cells) in 200 µl of PBS subcutaneously into the right side of the back of the animals. At five weeks after implantation, the animals were randomized into the following two groups; treatment group (n=11), intraperitoneally injected nafamostat mesilate  $(30 \ \mu g/g)$ three times a week for six weeks, and control group (n=13), intraperitoneal injection of the equal amount of vehicle three times a week for six weeks. The diameters of tumors in both groups were measured on the day of nafamostat mesilate or vehicle injection. At the end of the treatments, blood samples were taken, and animals were sacrificed. Subcutaneous tumors were excised, and the volume and weight were measured. Thereafter, paraformaldehyde-fixed and paraffin-embedded for immunohistochemistry and TdTmediated dUTP-X nick end labeling (TUNEL) assay.

Nucleic and whole protein were extracted from the tumor tissue for ELISA and Western blot analysis for assessment of inhibitory effect for NF- $\kappa$ B activity and apoptotic effect for cancer cells by nafamostat mesilate treatment. These *in vivo* experimental protocols were approved by the animal committee of Jikei University School of Medicine.

Histological studies. Paraffin sections of tumor tissue were stained immunohistochemically using NF- $\kappa$ B p65 monoclonal antibody (Epitomics, Burlingame, CA, USA, 1:500) as a primary antibody and Dako Envision Kit/HRP as a secondary antibody (Dako, Carpinteria, CA, USA) for evaluating NF- $\kappa$ B activation. TUNEL assay with In Situ Cell Death Detection Kit, TMR red (Roche Diagnostics, Indianapolis, IN, USA) was performed for evaluating induction of apoptosis. These assessments followed the manufacturer's instructions.

*Blood samples analysis.* The major adverse effects of nafamostat mesilate consist of hyperkalemia, hyponatremia and hepatopathy. To evaluate adverse effects, serum potassium, sodium, and alanine aminotransferase levels were measured.

*Statistical analysis*. Non-paired t-test and repeated measures ANOVA were used for statistical studies. All p-values were considered statistically significant when the associated probability was <0.05.

#### Results

Inhibition of NF- $\kappa$ B activity by nafamostat mesilate in vitro. In assessment of the NF- $\kappa$ B activity using ELISA, concentration of NF- $\kappa$ B p65 in the nuclear extracts of Panc-1 cells treated with nafamostat mesilate was significantly lower than those in control group (p=0.0001, Fig. 1a). In Western blot analysis, concentration of phosphorylated I $\kappa$ B $\alpha$  was lower, and concentration of I $\kappa$ B $\alpha$  was higher in Panc-1 cells treated with nafamostat mesilate, in comparison with those in control group (Fig. 1b). These results showed that NF- $\kappa$ B activity was inhibited due to suppressed I $\kappa$ B $\alpha$  phosphorylation in Panc-1 cells treated with nafamostat mesilate.

Inhibition of cell proliferation by nafamostat mesilate in vitro. In the cell proliferation assay, cell viability of Panc-1 cells treated with nafamostat mesilate was significantly lower than those in control group at each exposure time, including 24, 48 and 72 h ( $75.58\pm7.96\%$ , \*p=0.0030,  $56.47\pm4.97\%$ , \*\*p<0.0001,  $40.95\pm5.85\%$ , \*\*\*p<0.0001, Fig. 2). In addition, cell viability of Panc-1 cells treated with nafamostat mesilate was decreased exposure timedependently (p<0.0001).

Induction of apoptosis by nafamostat mesilate in vitro. In FACS analysis, cell counts of M1 period in nafamostat mesilate-treated cells were greater than those in control cells (Fig. 3a). In Western blot analysis, concentration of pro-caspase-8 was lower, and concentration of cleaved caspase-8 was higher in nafamostat mesilate-treated cells,



Figure 1. (a) Concentration of NF- $\kappa$ B p65 in the nuclear extracts of Panc-1 cells treated with nafamostat mesilate was significantly lower than those in control group. (b) In Western blot analysis, concentration of phosphorylated I $\kappa$ B $\alpha$  was lower, and concentration of I $\kappa$ B $\alpha$  was higher in Panc-1 cells treated with nafamostat mesilate, in comparison with those in control group.



Figure 2. Cell viability of Panc-1 cells treated with nafamostat mesilate was significantly lower than those in control group at each exposure time, including 24, 48 and 72 h.

in comparison with those in control cells (Fig. 3b). The results showed that nafamostat mesilate induced caspase-8 mediated apoptosis of Panc-1 cells, and increased the sub-G1 cell population.

Anti-tumor effect of in vivo nafamostat mesilate treatment. Fig. 4a shows tumors in both control and nafamostat mesilate-treated tumors at the end of study, respectively. As to



Figure 3. (a) Cell count of M1 period in nafamostat mesilate treatment cells was greater than those in control cells in FACS analysis. (b) Concentration of pro-caspase-8 was lower, and concentration of cleaved caspase-8 was higher in nafamostat mesilate-treated cells, in comparison with those in control cells in Western blot analysis.



Control





Nafamostat mesilate



Figure 4. (a) At the end of the study, nafamostat mesilate-treated tumors (arrow) were larger than control tumors (arrowhead). (b) The tumor growth in nafamostat mesilate-treated group was significant smaller than those in the control group. (c) The tumor volumes in nafamostat mesilate-treated group were significantly fewer than those in the control group. (d) The tumor weights in nafamostat mesilate-treated group were significantly fewer than those in control group.

changes in the tumor diameter, the initial diameter at five weeks after implantation in control group was 8.01±3.24 mm, which gradually grew to 17.93±4.45 mm at the end of six



Figure 5. (a) Concentration of NF- $\kappa$ B p65 measured by ELISA in the nuclear extracts of nafamostat mesilate-treated tumor was significantly lower than those in control. (b) Concentration of phosphorylated I $\kappa$ B $\alpha$  was lower, and concentration of I $\kappa$ B $\alpha$  was higher in nafamostat mesilate-treated tumors, in comparison with those in control in Western blot analysis. (c) In immunohistochemical staining, reduced nuclear translocation of NF- $\kappa$ B p65 was shown in nafamostat mesilate-treated tumors, in comparison with control tumors (x200).

weeks. In contrast, the tumor diameter in nafamostat mesilate treatment group was  $7.5\pm2.88$  mm, which gradually grew to  $12.89\pm4.27$  mm, but significant slower than those of the control group (p<0.0001) (Fig. 4b). Resected tumor volumes and weights in control group were  $2.779.5\pm1.658.48$  mm<sup>3</sup> and  $1.445.23\pm885.43$  mg, respectively. In contrast, those in the treatment group were  $1.190.78\pm903.49$  mm<sup>3</sup> and  $878.64\pm692.17$  mg. Both tumor volumes and weights in treatment group were significantly smaller than those of control group (p=0.0048, Fig. 4c, p=0.0496, Fig. 4d). These data showed that nafamostat mesilate treatment had inhibitory effect on the tumor growth of pancreatic cancer in an experimental mouse model.

Inhibition of NF- $\kappa$ B activity by nafamostat mesilate in vivo. In assessment of the NF- $\kappa$ B activity using ELISA, concentration of NF- $\kappa$ B p65 in the nuclear extracts of nafamostat mesilate-treated tumor was significantly lower than those in control (p=0.0383, Fig. 5a). In Western blot analysis, concentration of phosphorylated I $\kappa$ B $\alpha$  was lower, and concentration of I $\kappa$ B $\alpha$  was higher in nafamostat mesilate-treated tumors, in comparison with those in control (Fig. 5b). In immunohitochemical staining, reduced nuclear translocation of NF- $\kappa$ B p65 was shown in nafamostat mesilatetreated tumors (Fig. 5c). These results showed that NF- $\kappa$ B activity was inhibited due to suppressed I $\kappa$ B $\alpha$  phosphorylation in nafamostat mesilate-treated tumors *in vivo*, which is in accordance with the results of the *in vitro* experiment.

Induction of apoptosis by nafamostat mesilate in vivo. In Western blot analysis, concentration of pro-caspase-8 was lower, and concentration of cleaved caspase-8 was higher in nafamostat mesilate-treated tumors, in comparison with those in control (Fig. 6a). In TUNEL staining, TUNEL-positive cells were greater in nafamostat mesilate-treated tumors (Fig. 6b). These results showed that nafamostat mesilate treatment induced a caspase-8 mediated apoptosis *in vivo*, which is in accordance with the results of the *in vitro* experiment.

Adverse effects of in vivo nafamostat mesilate treatment. In post-therapeutic blood sample results, serum potassium levels of nafamostat mesilate-treated and control group were  $5.07\pm0.67$  mmol/l and  $4.6\pm0.16$  mmol/l, respectively (p=0.1160). Serum sodium levels of treatment and control



Figure 6. (a) In Western blot analysis, concentration of pro-caspase-8 was lower, and concentration of cleaved caspase-8 was higher in nafamostat mesilate-treated tumors, in comparison with those in control. (b) In TUNEL staining, TUNEL-positive cells were greater in nafamostat mesilate-treated tumors, in comparison with control (x100).



group were  $152\pm1.73$  mmol/l and  $152\pm2.45$  mmol/l, respectively (p=1.0000). Serum alanine aminotransferase levels of treatment and control group were 36±7.69 IU/l and 43.75±13.72 IU/l, respectively (p=0.2380). Blood test results related to known nafamostat mesilate adverse effects, such as hyperkalemia, hyponatremia and hepatopathy, were comparable between the two groups.

#### Discussion

NF-KB regulates the expression of genes involved in inflammatory cytokines, adhesion molecules and anti-apoptotic proteins. Therefore, the NF-kB inhibitor may suppress proliferation, invasion, metastasis and chemoresistance in tumors. Several reports described anti-tumor effect of protease inhibitor as an NF-KB inhibitor. Tsuzuki et al and Chih et al reported that nafamostat mesilate inhibited NF-κB activation in inflammation (28,29). Kimura et al reported that nafamostat mesilate inhibited liver metastasis and invasion of colon cancer cells in a mouse model (30), and Outa et al reported that nafamostat mesilate inhibited growth and invasion of pancreatic cancer cells by blocking tumor-associated trypsinogen into protease-activated receptor-2 in vitro (31,32). Uchima et al reported gabexate mesilate, a kind of protease inhibitors, prevented the invasive potential of pancreatic cancer cells and liver metastasis in nude mice (33). Yoon et al reported gabexate mesilate reduced the invasion and metastasis of colon cancer cells by inhibiting matrix metalloproteinases in vitro and in vivo (34).

Several drugs, which play roles as NF-κB inhibitors, have also been used for treatment of malignancies. However, these drugs have problems in clinical application, including

adverse effects. Bortezomib, which inhibits proteasome resulting in inhibition of NF-kB, is used for refractory and relapsed multiple myeloma in clinical medicine (35), but also inhibits reduction of unnecessary ubiquitin proteins. Curcumin, which is a yellow pigment present in the rhizome of turmeric has been shown to suppress NF-kB activation (36), and a phase II trial of curumin for advanced pancreatic cancer has been reported (37). However, curcumin is reported to impair function of tumor suppressor p53 in colon cancer cells (38). Resveratrol, a component of grapes, berries and peanuts, has been demonstrated to be a potent blocker of the NF-KB pathway (39) that enhances antitumor activity of gemcitabine in orthotopic mouse model of human pancreatic cancer (40). In the viewpoint of drug administration, resveratrol is available only orally, and has strong action for tumors in direct contact with the drug, such as skin and gastrointestinal tract tumors (41). Recent therapeutic advances in chemotherapy for malignancy allow patients with advanced cancer to live longer than before. Consequently, developments of therapeutic approach with less invasiveness and adverse effects for patients with poor general condition due to advanced cancer are needed to maintain and improve quality of life. Nafamostat mesilate is widely used for patients with poor performance status and organ function due to pancreatitis, disseminated intravascular coagulation or chronic renal failure, with minimal adverse effects. Therefore, nafamostat mesilate has a potential to become a new therapeutic option for cancer patients. In addition, because nafamostat mesilate is administrated via infusion, serum drug concentration is not affected by alimentary absorption.

Nafamostat mesilate

In conclusion, we demonstrated anti-tumor effect of nafamostat mesilate via inhibition of NF-KB activation by

suppression of  $I\kappa B\alpha$  phosphorylation, and induction caspase-8 mediated apoptosis *in vitro* and *in vivo* without definite adverse effects. Nafamostat mesilate treatment may be therapeutic application for patients with advanced pancreatic cancer.

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