Combination Chemotherapy of Nafamostat Mesilate with Gemcitabine for Pancreatic Cancer Targeting NF-κB Activation

TADASHI UWAGAWA[1], PAUL J. CHIAO[2], TAKESHI GOCHO[1], SHOUICHI HIROHARA[1], TAKEYUKI MISAWA[1] and KATSUHIKO YANAGA[1]

[1]*Department of Surgery, Jikei University School of Medicine, Tokyo, Japan;*
[2]*Department of Surgical Oncology, University of Texas M.D. Anderson Cancer Center, Houston, TX, U.S.A.*

Abstract. *Purpose: Gemcitabine is currently the standard first-line chemotherapeutic agent for pancreatic cancer. However, chemoresistance to gemcitabine because of gemcitabine-induced nuclear factor-κB (NF-κB) activation has been reported. We previously reported that the synthetic serine protease inhibitor nafamostat mesilate inhibited NF-κB activation and induced apoptosis of pancreatic cancer cells. In this study, whether or not nafamostat mesilate could enhance the anticancer effect of gemcitabine was investigated. Materials and Methods: NF-κB activation in pancreatic cancer cells treated with various agents was examined by electrophoretic mobility shift assay (in vitro) and immunohistochemistry by investigating the location of p65 in cancer cells (in vivo). Apoptosis of the cancer cells treated with agents was examined by flow cytometry. Results: Nafamostat mesilate inhibited gemcitabine-induced NF-κB activation, enhanced apoptosis by gemcitabine and suppressed pancreatic tumor growth. Interestingly, the combination treatment improved the body weight loss of mice induced by gemicitabine. Conclusion: This combination chemotherapy could be a potential novel strategy for pancreatic cancer.*

Pancreatic cancer is the fourth leading cause of cancer-related death with a 1-3% 5-year survival rate (1) and is characterized by a high incidence of loco-regional recurrence and distant metastasis. The prognosis of pancreatic cancer is extremely poor, and the most recommended treatment is surgical resection. However, only 25% of patients present with tumors amenable to resection because of the aggressive behavior of this type of cancer. Currently, for patients presenting with locally advanced or metastatic disease, chemotherapy with gemcitabine is the most potent and standard treatment (2, 3). Nevertheless, the tumor response rate of gemcitabine is below 10% and the median survival time is 5-6 months (4, 5), which is far from satisfactory (1) and new approaches to chemotherapy are needed. Beneficial effects have been reported with gemcitabine combined with erlotinib (6), or with capecitabine (7), however median survival times of each trial remained unacceptably low.

Constitutively activated nuclear factor-κB (NF-κB) that promotes cell proliferation and regulates the gene involved in invasion (8) and angiogenesis (9, 10) plays a key role in the aggressive behavior of pancreatic cancer (11). Some chemotherapeutic agents, including gemcitabine, induce NF-κB activation in cancer cells, and several studies have shown that chemotherapeutic agent-induced NF-κB activation is a key mediator of chemoresistance (12-16). We previously reported that nafamostat mesilate, an effective therapeutic agent for disseminated intravascular coagulation, systemic inflammatory response syndrome and pancreatitis (17, 18), inhibited NF-κB activation by suppressing I kappa B (IκB) kinase-mediated IκBα phosphorylation and induced apoptosis through disruption of interconnected signaling pathways by both suppressing NF-κB antiapoptotic activity and inducing tumor necrosis factor receptor (TNFR)-mediated apoptosis (19). Based on this, we hypothesized that nafamostat mesilate may inhibit gemcitabine-induced NF-κB activation and as a result, enhance the apoptosis induced by gemcitabine.

In this report, the anticancer effect of combination chemotherapy of gemcitabine with nafamostat mesilate for pancreatic cancer was compared with treatment with gemcitabine alone through both *in vitro* and *in vivo* study.

Materials and Methods

Reagents. Nafamostat mesilate was donated by Torii Pharmaceutical (Tokyo, Japan) and was stored in a stock solution (5 mg/ml) in sterile water at –20˚C. Radioisotopes were obtained from Amersham Biosciences.

Correspondence to: Tadashi Uwagawa, Department of Surgery, Jikei University School of Medicine, 3-25-8 Nishi-shinbashi, Minato-ku, Tokyo 105-8461, Japan. Tel: +81 334331111 Ext. 3401, Fax: +81 354724140, e-mail: uwatadashi@msn.com

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Cell culture. The human pancreatic tumor cell line Panc-1 (ATCC, Rockville, MD, USA) was maintained in DMEM supplemented with 10% heat-incubated fetal bovine serum. The cells were incubated at 37˚C in a humidified 5% CO_2 atmosphere.

Electrophoretic mobility shift assay (EMSA). The EMSA and preparation of nuclear extracts were performed as described elsewhere (10). Briefly, after the incubation of 15 μg of nuclear extract in a 10 μl reaction volume containing 75 mM NaCl; 15 mM Tris-HCl, pH 7.5; 1.5 mM EDTA; 1.5 mM DTT; 25% glycerol; 20 μg/ml BSA and 1 μg poly (dI-dC) on ice for 40 min, end-labeled DNA probes (kB: 5'-AGTTGAGGGGACTTTCCCAGGC-3' and Oct-1: 5'-TGTCGAATGCAAATCACTAGAA-3') were added to the reaction mixture and incubated at room temperature for 20 min and then applied to a 4% nondenatured polyacrylamide gel containing 0.25×TBE (22.5 mM Tris, 22.5 mM borate, 0.5 mM EDTA; pH 8.0) buffer. Equal loading of the nuclear extracts was monitored by Oct-1 binding. After electrophoresis, the gel was dried and exposed to X-ray films at –80˚C.

Cell cycle analysis. Panc-1 cells were treated with gemcitabine for 48 hours with or without pretreatment with nafamostat mesilate (80 μg/ml) for 3 hours. Control cells remained untreated with these agents. The cell cycle was analyzed by flow cytometry. In brief, the cells were harvested and 1×10^6 were fixed with 70% (v/v) ethanol for 24 h at –20˚C. After centrifugation, the cell pellet was washed with PBS (pH 7.4) and resuspended in PBS containing propidium iodode (50 μg/ml), Triton® X-100 (0.1% , v/v), 0.1% sodium citrate and DNase-free RNase (1 μg/ml). The cells were incubated at room temperature for 30 min and the DNA content was determined with a FACScan flow cytometer (Becton Dickinson, MD, USA).

Experimental animals. Five-week-old male nude mice (ICR-SCID) were purchased from CLEA Japan Inc. (Tokyo, Japan). Panc-1 cells were suspended in PBS, and 5×10^6 cells in 100 μl were implanted subcutaneously into the back of each animal. Five weeks after implantation, the mice were randomized into the following treatment groups: untreated control (n=9); gemcitabine alone (100 mg/kg), once weekly by *i.p.* injection (n=10); combination of gemcitabine (100 mg/kg) once weekly by *i.p.* injection, with nafamostat mesilate (30 mg/kg) thrice weekly by *i.p.* injection (n=10). The gemcitabine was dissolved in 100 μl PBS and the nafamostat mesilate in 100 μl sterile water. After six weeks of treatment, the animals were sacrificed and weighed. The subcutaneous tumors were excised and the tumor volume was measured as length×width×thickness. The tumor tissue was formalin-fixed and paraffin-embedded for immunohistochemistry and routine H&E staining. All of the *in vivo* experimental protocols were approved by the Animal Committee of Jikei University School of Medicine.

Immunohistochemical staining. The paraffin-embedded pancreatic tumors were cut and mounted on slides and then washed in PBS. The slides were blocked with protein block solution (DakoCytomation, Denmark) for 20 min and incubated overnight with rabbit polyclonal anti-human p65 (1:100). After the incubation, the slides were washed and incubated with biotinylated link universal antiserum followed by horseradish peroxidase-streptavidin conjugate (LSAB+ kit). The slides were rinsed, and color was developed using 3,3-diaminobenzidine hydrochloride as chromogen. Finally, sections were rinsed in distilled water, counterstained with Mayer's hematoxylin, and mounted with DPX mounting medium for evaluation. To evaluate NF-κB activation in the Panc-1 pancreatic cancer cells, the number of nuclear p65-positive cells was counted in three randomly selected high-power fields (×400).

Statistics. Statistical analysis was performed with statview-J5.0 software. Results are expressed as mean±SE. $P<0.05$ was considered to be significant.

Results

Effect of nafamostat mesilate on gemcitabine-induced NF-κB activity. The NF-κB DNA-binding activity in the nuclear extracts from the Panc-1 cells was investigated by EMSA. Nafamostat mesilate inhibited the NF-κB activation that is constitutively activated in Panc-1 cells (Figure 1a). Gemcitabine induced NF-κB activation and the maximum levels were observed 3 hours after the treatment (Figure 1b). Nafamostat mesilate effectively inhibited the gemcitabine-induced NF-κB activation (Figure 1c).

Effect of gemcitabine with nafamostat mesilate on apoptosis in Panc-1 cells. The effect of combination treatment with gemcitabine and nafamostat mesilate on apoptosis (sub G_0/G_1 phase) was assessed by flow cytometry. The largest percentage of the population in the sub G_0/G_1 phase was observed in the gemcitabine/nafamosat mesilate group (control 7.82%; gemcitabine 11.27%; gemcitabine/nafamostat mesilate 14.56%), suggesting that nafamostat mesilate enhanced apoptosis by gemcitabine (Figure 2).

Effect of combination treatment in vivo on pancreatic cancer tumors. In comparison with the control, the combination treatment suppressed tumor growth significantly ($p<0.05$). On the other hand, while gemcitabine alone reduced the tumor size, the difference from the controls was not significant (Figure 3).

Effect of nafamostat mesilate on NF-κB activation in vivo. Since the localization of NF-κB in the tumor cells reflects NF-κB activity, the localization of p65, a subunit of its was investigating by immunohistochemistry. Nafamostat mesilate induced reduced nuclear translocation of p65 (Figure 4a,b), suggesting that nafamostat mesilate also inhibited gemcitabine-induced NF-κB activation of pancreatic cancer cells *in vivo* .

Effect of nafamostat mesilate on gemcitabine-induced body weight loss. Interestingly, gemcitabine induced significant body weight loss in comparison with the other two groups (Figure 5). No significant difference was observed between the control and the combination treatment. However, the tumor weight in the control group also increased (Figure 3). These results suggested that nafamostat mesilate has the potential to improve malnutritional induced by gemcitabine or pancreatic cancer.

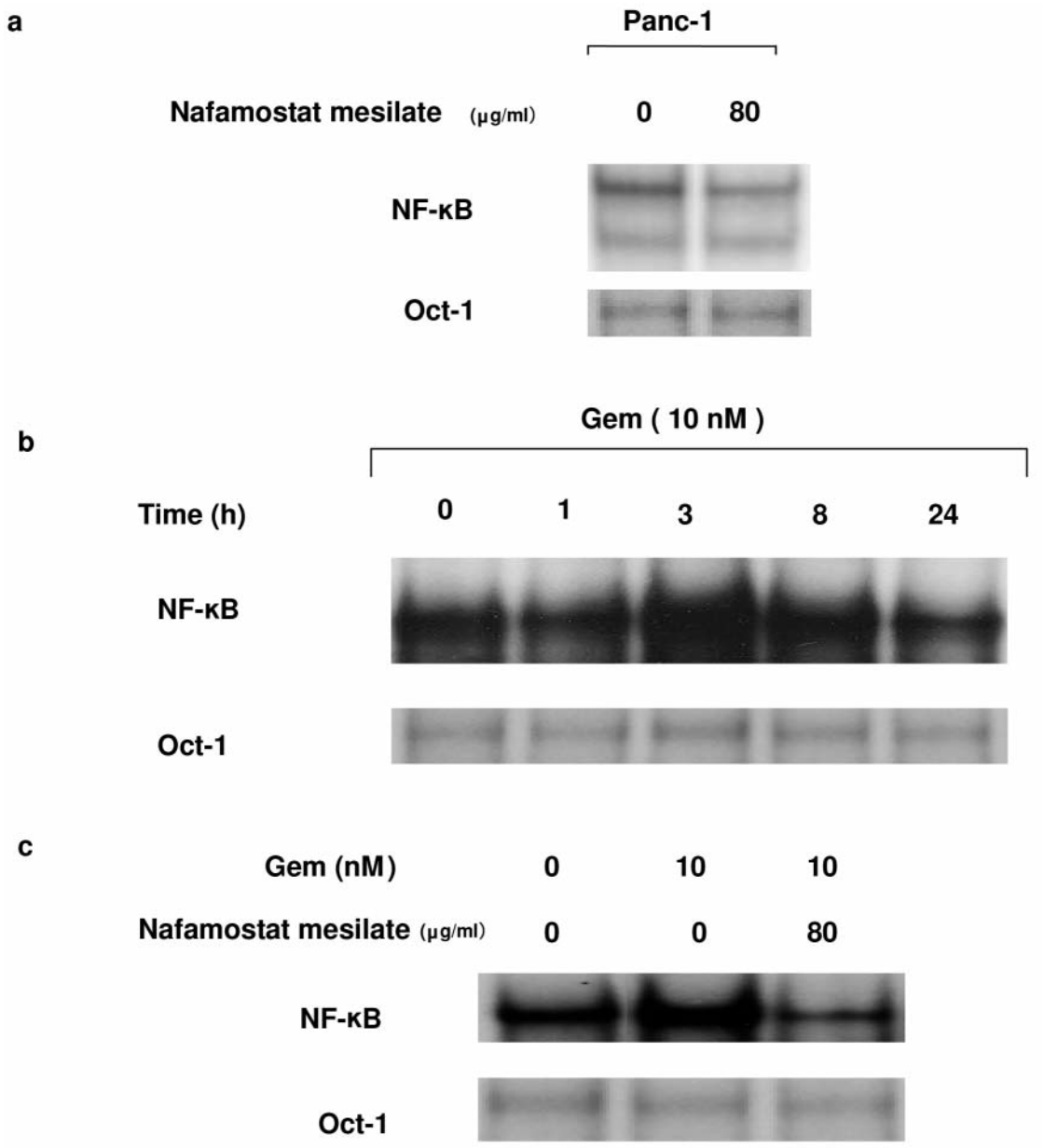

Figure 1. *Effect of nafamostat mesilate on gemcitabine-induced NF-κB activation in Panc-1 pancreatic cancer cells. Nuclear extracts from cells treated (a) with or without nafamostat mesilate for 24 h, (b) with gemcitabine for various periods, or (c) pretreated with or without nafamostat mesilate for 3 hours, and then treated with or without gemcitabine for 3 hours were subjected to electromobility shift assay (EMSA) with a κB probe. Oct-1 was used as loading control.*

Discussion

Gemicitabine is characterized by a mechanism of action which includes cytotoxic self-potentiation, masked DNA chain termination and potent inhibition of DNA repair, and the induction of apoptosis in pancreatic cancer cells (20). At the same time, however, gemcitabine also induces NF-κB activation.

To initiate NF-κB activation, the sequential activation of IκB kinases (IKKs) that induce proteasomal degradation of IκBα proteins through phosphorylation of IκBα is needed. Once IKKs are activated, by a variety of stimuli, and after the proteasomal degradation of IκBα, the NF-κB complexes bind with IκBα in the cytoplasm and translocate into the nucleus where NF-κB binds to a promoter of the target gene. Kunnumakkara *et al*. reported that the NF-κB

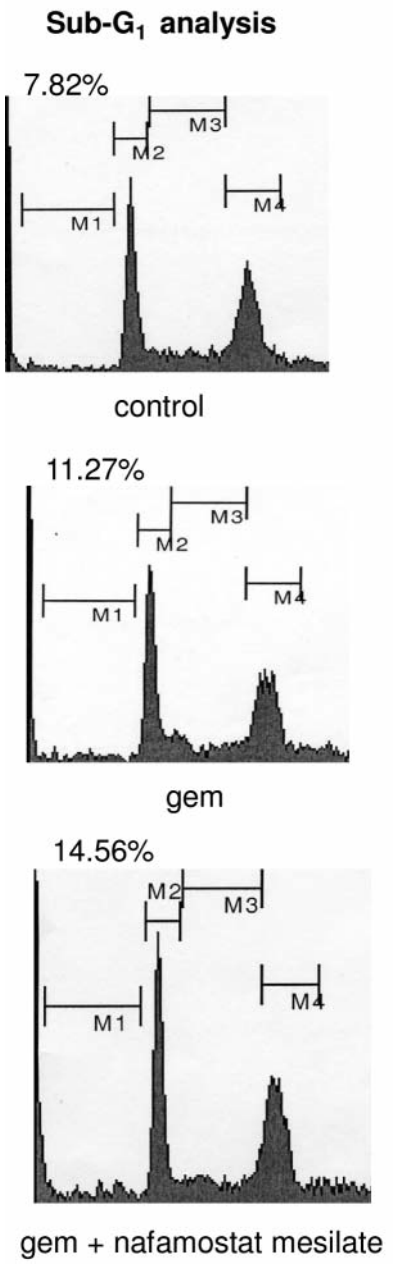

Figure 2. *Effect of nafamostat mesilate on gemcitabine-induced apoptosis in Panc-1 cells. Cells were pretreated with or without nafamostat mesilate (80 μg/ml) for 3 hours, and then treated with gemcitabine (gem)(10 nM) for 48 hours and subjected to flow cytometry to determine cell cycle profiles and SubG$_1$ cell populations. Control: no treatment; M1: sub-G1; M2: G0/G1; M3: S; M4: G2/M.*

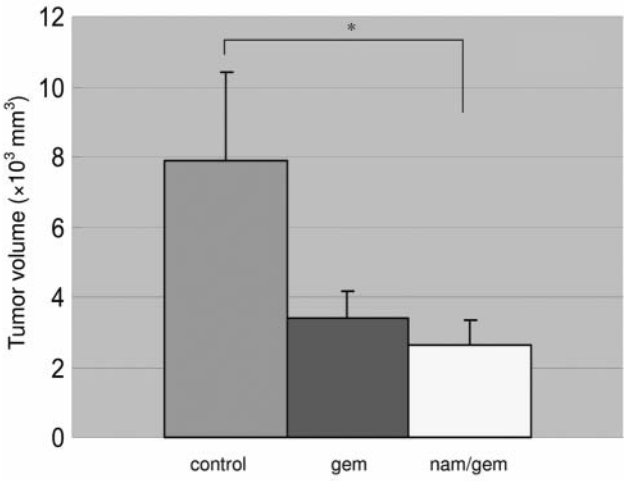

Figure 3. *Effect of combination treatment of nafamostat mesilate with gemcitabine on pancreatic tumor growth. Five weeks after Panc-1 cells were implanted into subcutaneously into nude mice, treatments with gemcitabine alone (100 mg/kg), once weekly) or combination gemcitabine (100 mg/kg, once weekly) with nafamostat mesilate (30 mg/kg, thrice weekly) or control, no treatment, were administered for six weeks. The animals were sacrificed and the resected tumor size (length×width×height) measured. *p<0.05.*

inhibitor curcumin potentiated the antitumor activity of gemcitabine (21). Banerjee *et al*. showed that genistein inhibited gemcitabine-induced NF-κB activation, and as a result enhanced chemosensitivity (22). In the present study, a novel combination treatment with the NF-κB inhibitor synthetic serine protease inhibitor, nafamostat mesilate and gemcitabine induced apoptosis in pancreatic cancer cells effectively by increasing chemosensitivity to gemcitaine as a result of inhibiting gemcitabine-induced NF-κB activity. Furthermore, the combination of gemcitabine with nafamostat mesilate, not gemcitabine alone, suppressed the growth of pancreatic cancer cells significantly, suggesting that this combination therapy had a synergistic anticancer effect. Although the effect of nafamostat mesilate on various cells remains unclear, the most interesting point is that nafamostat mesilate has a function not only as an NF-κB inhibitor, but also as an activator of the caspase-8 signaling pathway, which plays an important role in proapoptotic signal transduction, through the up-regulation of TNFR1 induced by transcription factor PEA3 (19). Thus, nafamostat mesilate has dual functions as an inhibitor of antiapoptotic and an activator of pro-apoptotic signal transduction, which might be a major mechanism for enhancing the cell death induced by gemcitabine in pancreatic cancer. Furthermore, the dose of nafamostat mesilate used in this study is relevant to the clinical dose used for disseminated intravascular coagulation in Japan, suggesting that this combination treatment could easily be applicable to clinical practice. Chemotherapeutic agents often cause various adverse effects in clinical treatment. Gastrointestinal adverse effects, such as anorexia, constipation or diarrhea often result in undernourishment, and body weight could be a good indicator of nutritional

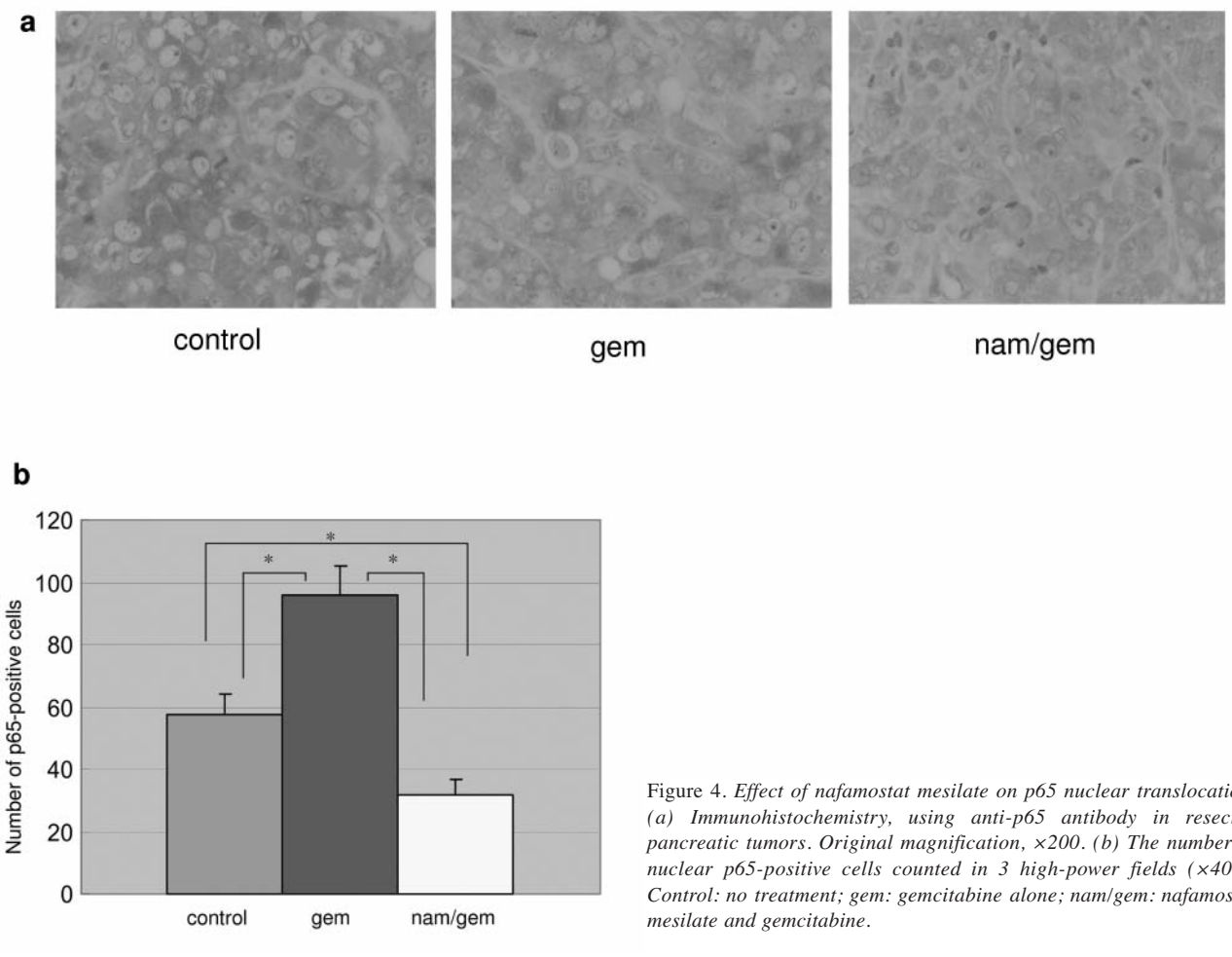

Figure 4. *Effect of nafamostat mesilate on p65 nuclear translocation. (a) Immunohistochemistry, using anti-p65 antibody in resected pancreatic tumors. Original magnification, ×200. (b) The number of nuclear p65-positive cells counted in 3 high-power fields (×400). Control: no treatment; gem: gemcitabine alone; nam/gem: nafamostat mesilate and gemcitabine.*

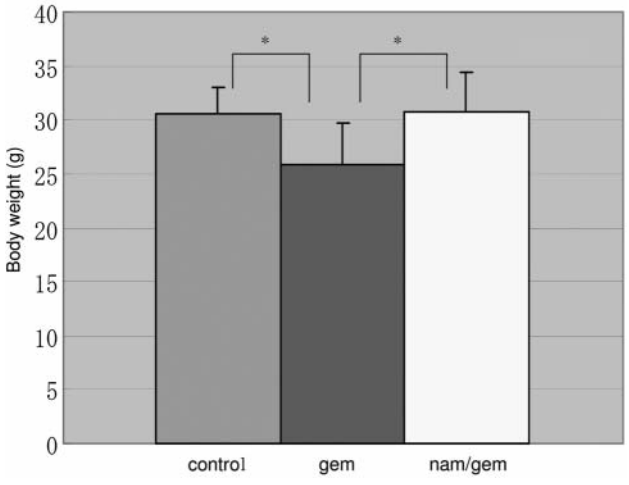

Figure 5. *Effect of nafamostat mesilate on gemcitabine-induced body weight loss of mice. Body weight after six weeks of treatment, gem; gemcitabine alone (100 mg/kg, once weekly),nam/gem; combination of gemcitabine (100 mg/kg, once weekly) with nafamostat mesilate (30 mg/kg, thrice weekly) or control; no treatment. *p<0.05.*

status. Although a mechanism through which nafamostat mesilate inhibited the body weight loss induced by gemcitabine could not be pinpointed, this effect would be advantageous in clinical medicine, and would suggest improved quality of life. Based on our results, the combination of gemicitabine with nafamostat mesilate targeting NF-κB activation may be a novel combination chemotherapy for pancreatic cancer.

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