

Nafamostat mesylate prevents metastasis and dissemination of neuroblastoma through vascular endothelial growth factor inhibition

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Abstract. Neuroblastoma is a highly malignant disease with a poor prognosis and few treatment options. Despite conventional chemotherapy for neuroblastoma, resistance, invasiveness, and metastatic mobility limit the treatment efficacy. Therefore, it is necessary to develop new strategies for treating neuroblastoma. The present study aimed to evaluate the anticancer effects of nafamostat mesylate, a previously known serine protease inhibitor, on neuroblastoma cells. Effects of nafamostat mesylate on neuroblastoma cell migration and proliferation were analyzed by wound healing assay and WST-8 assay, respectively. To elucidate the mechanisms underlying the effects of nafamostat mesylate on neuroblastoma, the expression levels of NF- κ B were measured via western blotting, and the production of the cytokine vascular endothelial growth factor (VEGF) in the cell culture supernatants was determined via ELISA. In addition, a mouse model of hematogenous metastasis was used to investigate the effects of nafamostat mesylate on neuroblastoma. It was determined that nafamostat mesylate significantly inhibited migration and invasion of Neuro-2a cells, but it had no effect on cell proliferation at 24 h after treatment. Exposure of Neuro-2a cells to nafamostat mesylate resulted in decreased vascular endothelial growth factor production, which could be a pivotal mechanism underlying the inhibitory effects of neuroblastoma metastasis. The results of the present study suggest that nafamostat mesylate may be an effective treatment against neuroblastoma invasion and metastasis.

Introduction

Neuroblastoma is the most common extracranial solid tumor in children; approximately half of patients with

neuroblastoma present with metastatic disease at the time of diagnosis (1,2). Metastasis of neuroblastoma can occur by the hematogenous route, seeding bone marrow, liver, and bone. The treatment of patients with disseminated neuroblastoma is one of the greatest challenges for pediatric oncologists, given that the five-year survival rate remains as low as 40-45%, despite advanced treatment options (3). Therefore, it is essential to develop effective strategies to inhibit tumor metastasis.

Cancer metastasis begins with the detachment of metastatic cells from the primary tumor, migration of the cells to distal sites through blood vessels, settlement, and growth at the distal site (4). During this process, metastatic cells go through detachment, migration, invasion, and adhesion (4). Each of these processes involves rate-limiting steps influenced by non-malignant cells of the tumor microenvironment (5). Tumor vascularization is a rate-limiting step for metastasis. Vascular endothelial growth factor (VEGF) is a highly potent molecule that increases vessel permeability, endothelial cell growth, proliferation, migration, and differentiation (6).

Nafamostat mesylate is a synthetic serine protease inhibitor approved for pancreatitis and disseminated intravascular coagulation (DIC) (7). This agent inhibits thrombin, plasmin, kallikrein, trypsin, and C1 esterase in the complement system and factors VIIa, Xa, and XIIa in the coagulation cascade. Moreover, nafamostat mesylate inhibits the metastasis of colon cancer, pancreatic cancer, breast cancer, and squamous cell carcinoma (8-14). However, the effects of nafamostat mesylate on other types of cancer have been less extensively studied.

In the present study, the effects of nafamostat mesylate on neuroblastoma regarding cell proliferation, motility, cell-invasive potential, and growth factor production were investigated. It is suggested that the results of the present study will contribute to developing new aspects of treatment for neuroblastoma.

Materials and methods

Reagents. Nafamostat mesylate was obtained from Nichi-Iko Pharmaceutical Co., Ltd. (product no. 873999), dissolved in distilled water, and stored at -80°C until use.

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Cell culture. The murine neuroblastoma cell line, Neuro-2a (cat. no. ATCC-CCL-131; American Type Culture Collection), was maintained in RPMI-1640 medium (product no. R8758; Sigma-Aldrich; Merck KGaA) and 10% fetal bovine serum (FBS; cat. no. CCP-FBS-BR-500; Cosmo Bio Co., Ltd.). The cells were cultured in a standard T75 cell culture flask (cat. no. 156499; Thermo Fisher Scientific, Inc.) at 37°C with 5% CO₂ to a confluency of 80%. The Neuro-2a cells were then subcultured to 80% confluency, and cells passages three to eight were used for the experiments. The doubling time was 28.9 h and the doubling rate of the cells remained relatively constant through passages three to eight.

Cell proliferation assay. The effects of nafamostat mesylate on cell growth was evaluated using the WST-8 Cell Counting reagent (cat. no. 341-07761; Dojindo Molecular Technologies). A total of 2×10^3 Neuro-2a cells were seeded in 100 μ l RPMI-1640+10% FBS on 96-well plates which were pre-incubated for 24 h before the addition of a 50- μ M concentration of nafamostat mesylate. WST-8 (10 μ l) was added to each well at a 1:10 ratio in a cell culture medium. After 2.5 h of incubation in a humidified atmosphere at 37°C with 5% CO₂, the absorbance at 450 nm was measured using a spectrophotometer (cat. no. 51119000; Thermo Fisher Scientific, Inc.). The results are expressed as the means \pm SD from three independent experiments.

Wound healing assay. Neuro-2a cells (5×10^6 cells) were seeded onto a 6-well plate. A 90% confluent monolayer of cells was then scratched with a pipette tip and subsequently washed with media to remove the floating cells. In order to examine whether nafamostat inhibited the migration of Neuro-2a cells induced by serum, the cells were then incubated with two concentrations (10 and 50 μ M) of nafamostat mesylate in RPMI-1640+10% FBS as previously described (15), and images of the cell migration into the wound at 24 h were captured by light microscope (15).

Quantification of cytokine secretion by enzyme-linked immunosorbent assay (ELISA). A total of 1.5×10^5 Neuro-2a cells were seeded onto a 6-well plate. After 24 h of incubation, the cells were treated with 50 μ M nafamostat mesylate for 24 h. Following harvesting of the culture supernatants, the VEGF levels were analyzed by ELISA using the Quantikine kit (cat. no. MMV00; R&D Systems). All experiments were performed at least three times, and the mean was calculated.

Western blotting. Cytoplasmic extracts were obtained as previously described (16). Briefly, Neuro-2a cells (1×10^7 cells) were resuspended in 1 ml of RIPA buffer (cat. no. 08714-04; Nakalai Tesque, Inc.) at 4°C for 30 min, and then the cells were centrifuged (8,000 \times g) at 4°C for 15 min. The proteins (40 μ g/lane), quantified using bicinchoninic acid assay (BCA), were electrophoresed on a 10% sodium dodecyl sulfate polyacrylamide gel followed by semi-dry transfer to a polyvinylidene fluoride (PVDF) membrane (cat. no. 88518; Invitrogen; Thermo Fisher Scientific, Inc.). The transferred PVDF blots were pretreated with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature and incubated with primary antibodies for nuclear factor- κ B (NF- κ B;

product no. 8242; 1:3,000; Cell Signaling Technology, Inc.) and actin (cat. no. A2066; 1:3,000; Sigma-Aldrich; Merck KGaA) at 4°C overnight. The membranes were then washed three times with TBST and incubated with horseradish peroxidase-conjugated secondary antibody (product code ab99697; 1:3,000; Abcam) for 1 h at room temperature. Following washing three times again, the antibodies bound to the protein blots were detected using Western Lightning Plus Chemiluminescence Reagent (cat. no. NEL103E001EA; PerkinElmer Life Sciences), visualized on a LAS-3000 Mini Digital Imaging System (FUJIFILM Corporation).

Animals. Female, eight-week-old A/J mice (n=18, weight 17–21 g), which express the MHC haplotype H-2^a, were purchased from Japan SLC, Inc. The mice were maintained in specific pathogen-free conditions. All animal procedures and experiments were performed according to a protocol approved by the Animal Ethics Committee (Permission number 27-34), Mie University Graduate School of Medicine, Tsu, Mie, Japan.

In vivo experimental animal models. The mouse neuroblastoma dissemination model was established by injecting Neuro-2a cells (1×10^6 cells) into the lateral tail vein of A/J mice (17). Two groups, a nafamostat-treated group (n=12) and a control group (n=6), were prepared as follows: The nafamostat-treated group was intravenously injected with Neuro-2a cells incubated with nafamostat mesylate (50 μ g/ml) for 24 h. The control group was injected with Neuro-2a cells incubated with a vehicle for 24 h. The mice were intravenously injected with Neuro-2a cells on day 0. The progression of internal tumors was monitored daily by assessing survival, clinical conditions and body weight. Animals were euthanized by cervical dislocation if they lost >20% of their original body weight, if they lost weight rapidly (>1 g per day for two consecutive days), or if they became morbid or exhibited the first sign of any distress.

Statistical analysis. The normality of the distribution was assessed using BellCurve (Excel software; Social Survey Research Information Co., Ltd.). Unpaired Student's t-tests were used to analyze significant differences between two groups. Data are expressed as the mean \pm standard deviation. The survival analysis was performed using Kaplan-Meier curves along with log-rank testing. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of nafamostat mesylate on neuroblastoma cell migration and proliferation. The majority of patients with neuroblastoma develop metastatic disease at diagnosis and their prognosis is poor with current therapeutic approaches (18). To investigate the effects of nafamostat mesylate on cell motility, a 90% confluent cell layer was scratched with a pipette tip and treated with nafamostat mesylate in RPMI-1640+10% FBS for 24 h. As revealed in Fig. 1A, nafamostat mesylate markedly reduced the migration of Neuro-2a cells into the scratched area over 24 h compared with the untreated group in a dose-dependent manner. Previous studies have demonstrated the

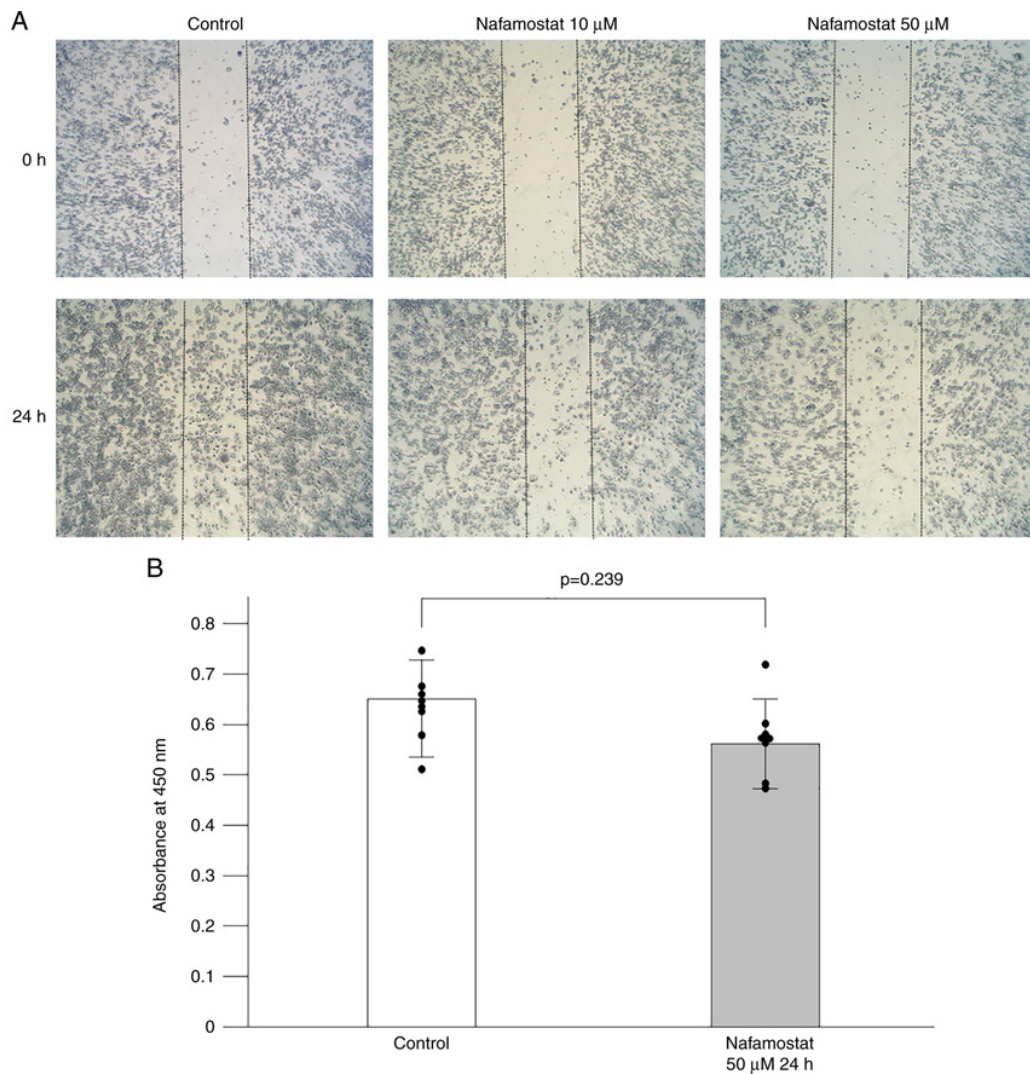


Figure 1. Nafamostat mesylate inhibits cell migration and invasion. (A) Wound healing assays were performed. Neuro-2a cells were cultured in 6-well plates until they formed a 90% confluent monolayer. The monolayer was scratched with a pipette tip, and the cells were treated with nafamostat mesylate (50 μ M, nafamostat-treated group) or with the vehicle only (distilled water, control group). Images of cell migration into the wound were captured at 0 and 24 h after wound initiation. Magnification, x40. (B) Cell viability was measured using a WST-8 assay 24 h after treatment. Unpaired Student's t-tests were used to analyze significant differences between the two groups. No significant difference was observed between the nafamostat-treated group and the control group.

inhibitory effect of nafamostat mesylate on cell proliferation in pancreatic, colon, and breast cancers (9-11). To determine whether nafamostat mesylate affected neuroblastoma cell proliferation, a WST-8 assay was performed. Neuro-2a cells were exposed to a 50- μ M concentration of nafamostat mesylate for 24 h and were then assessed using the WST-8 assay. The cell proliferation assay revealed that nafamostat mesylate may not affect cell proliferation because nearly the same absorbance was observed between the nafamostat-treated and the vehicle-treated groups at the 24-h interval (Fig. 1B).

Inhibition of neuroblastoma dissemination by nafamostat mesylate *in vivo*. In view of the ability of nafamostat mesylate to potentially inhibit Neuro-2a cell migration, the effects of nafamostat mesylate *in vivo* were investigated. Neuroblastoma hematogenously metastasizes to bone marrow, liver, skin, and bone. To model hematogenous metastasis, murine neuroblastoma Neuro-2a cells were intravenously injected into the lateral tail vein of A/J mice as previously described (17). Consequently,

multiple liver nodules were observed (17). Nafamostat mesylate-treated Neuro-2a cells (nafamostat-treated group) or vehicle-treated Neuro-2a cells (control group) were intravenously injected into the tail veins of A/J mice. Representative images of the liver nodules and mesentery metastasis in the control group at euthanasia or death after injection of Neuro-2a cells are presented in Fig. 2A. Liver metastasis was present in all animals in both the control and nafamostat-treated groups at euthanasia or death. Approximately 80% of the animals had at least a 10% weight loss (Fig. 2B). Survival analysis was performed using Kaplan-Meier with log-rank testing, for animals with experimental liver dissemination by Neuro-2a, and the median survival times in the nafamostat-treated and the control groups were 56 and 26 days, respectively. The survival time of the nafamostat-treated group was statistically longer than that of the control group ($P < 0.05$, log-rank test).

Effects of nafamostat mesylate on Neuro-2a cells. Angiogenesis, the formation of new blood vessels, plays a

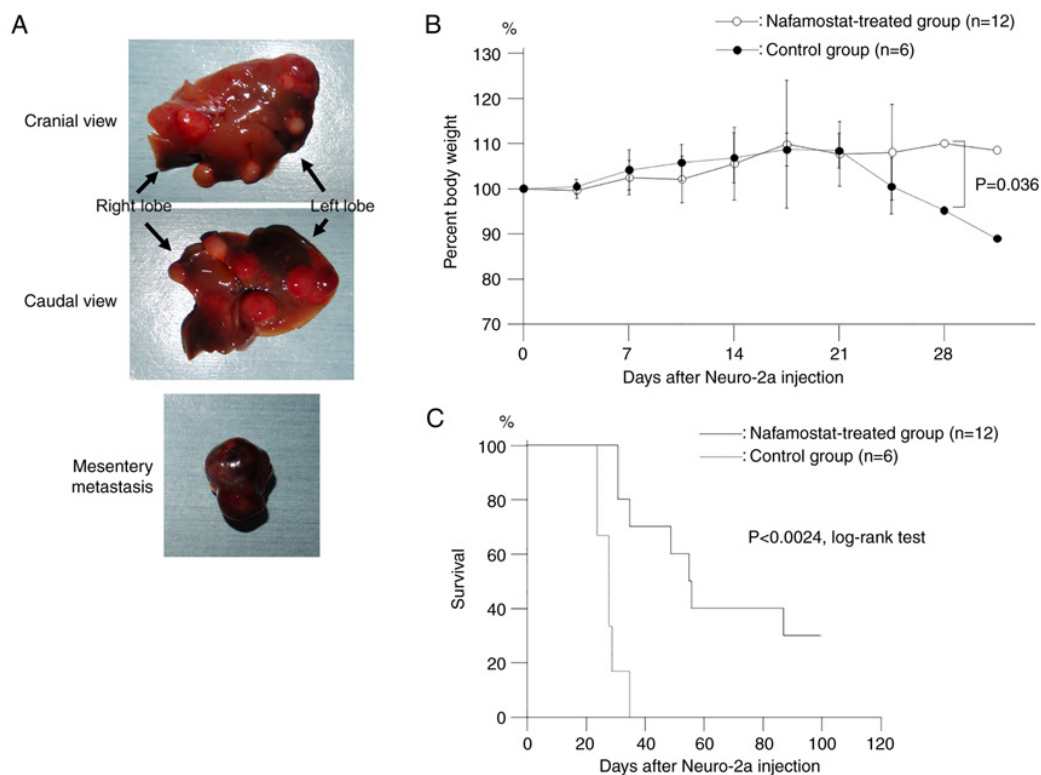


Figure 2. Nafamostat mesylate inhibits the dissemination of Neuro-2a cells in A/J mice. (A) Growth of intravenously implanted tumors. Liver and mesentery metastases were extracted from a mouse who received an intravenous injection of Neuro-2a cells. (B) The weight of the mice in the nafamostat-treated group and control group was evaluated at each time-point. Unpaired Student's t-tests were used to analyze significant differences between the two groups. (C) The survival rate of the mice after intravenous Neuro-2a injection. The length of survival in the nafamostat-treated group was significantly longer than that in the control group ($P<0.05$, log-rank test).

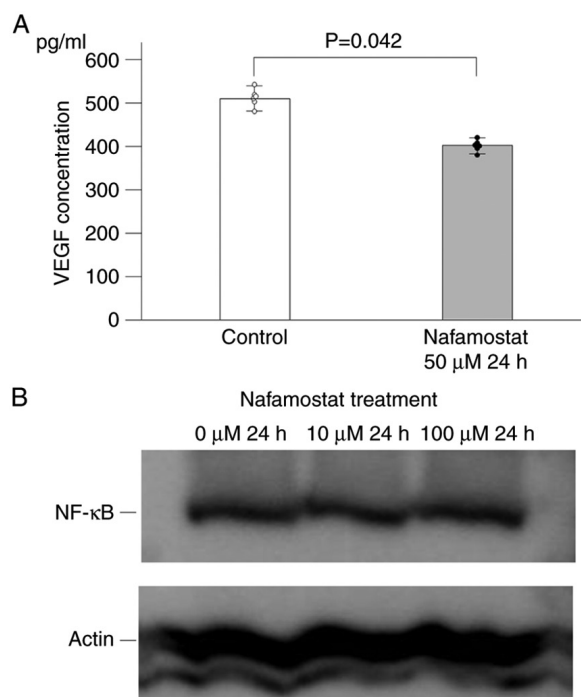


Figure 3. Effects of nafamostat mesylate on Neuro-2a cells. (A) Neuro-2a cells were incubated with nafamostat mesylate (0 or 50 μ M) for 24 h. The VEGF concentrations in cell culture supernatants were measured using ELISA. Unpaired Student's t-tests were used to analyze significant differences between the two groups. (B) Neuro-2a cells were incubated with nafamostat mesylate (0, 10, or 100 μ M) for 24 h. The expression levels of NF- κ B (top) and actin (bottom) were detected using western blotting. VEGF, vascular endothelial growth factor; NF- κ B, nuclear factor- κ B.

central role in the process of tumor growth and metastasis. VEGF has been confirmed as the most potent inducer of angiogenesis (6). To investigate the effects of nafamostat mesylate on angiogenesis, the concentration levels of VEGF in the cell culture supernatants were evaluated by ELISA. In the presence of nafamostat mesylate, the concentration of VEGF in the culture supernatant was significantly decreased for Neuro-2a cells (Fig. 3A). Inhibition of NF- κ B by nafamostat mesylate has been reported to suppress VEGF expression (8,12). To determine whether nafamostat mesylate inhibits the activity of the NF- κ B pathway, western blot analysis was performed to assess the expression levels of NF- κ B in Neuro-2a cells after nafamostat mesylate treatment. The results indicated that nafamostat mesylate did not inhibit the expression levels of NF- κ B (Fig. 3B).

Discussion

Neuroblastoma is usually highly aggressive in patients over one year of age and leads to poor outcomes with a high percentage of metastatic cases (19). Therefore, suppressing tumor cell migration and invasion could be a unique therapeutic approach to inhibit metastasis. Nafamostat mesylate is a synthetic serine protease inhibitor that has been used to treat DIC and acute pancreatitis. Although the anticancer activity of nafamostat was demonstrated in several cancers (8,10-12,14), the effect of nafamostat mesylate on neuroblastoma has not yet been explored. The present study demonstrated that nafamostat mesylate suppressed neuroblastoma cell dissemination

by inhibiting VEGF production from neuroblastoma cells. Moreover, nafamostat mesylate did not affect neuroblastoma cell proliferation at 24 h.

The anticancer activity of nafamostat mesylate is still controversial. Nafamostat mesylate reportedly inhibited cell proliferation and cell invasion in colorectal cancer and squamous cell carcinoma via inhibition of the NF- κ B (10,14). However, Saito *et al* (12) and Fujiwara *et al* (8) reported that nafamostat mesylate suppressed cell adhesion and invasion, but not cell viability, in pancreatic cancer by downregulating NF- κ B levels. Although NF- κ B upregulation in cancer cells is the main stimulator of cell proliferation, nafamostat mesylate did not alter the expression level of NF- κ B in Neuro-2a cells at 24 h (data not shown). This result suggests that nafamostat mesylate inhibits cell proliferation with cancer-type or cell-type specificity and does not regulate the signaling pathways related to cell proliferation in certain types of cancer.

Investigating the mechanisms of cancer invasion and designing tools targeted at cancer cell metastasis in combination with current therapies could open up new possibilities to reduce mortality in highly invasive neuroblastoma cases. A recent breakthrough study by Kitchen *et al* highlighted the importance of re-purposing drugs that target signaling pathways to treat central nervous system (CNS) edema when there are no therapeutic options available. They demonstrated that the translocation of water channel protein aquaporin-4 (AQP4) to the surface of astrocyte cells was mediated by protein kinase A (PKA) and calmodulin (CaM), resulting in CNS edema due to increased water flux (20). Furthermore, the CaM inhibitor trifluoperazine, which is used for treating schizophrenia, was reported to effectively reduce CNS edema by inhibiting AQP4 localization on the cell surface (20,21). Existing approved drug, nafamostat mesylate, may be re-purposed for the treatment of disseminated neuroblastoma for which there is no effective therapeutic option available. Further studies should evaluate the molecular mechanisms of nafamostat mesylate treatment for inhibition of neuroblastoma metastasis.

Targeting the molecular and signaling mechanisms rather than only using traditional approaches is important to meet the urgent, unmet clinical needs of a number of patients for whom no pharmacological interventions are available. AQP1 is predominantly expressed in the brain, and plays a role in the migration of neural crest cells, with differential AQP1 levels affecting migration speed, direction, and filopodia length (22-24). Neuroblastoma, which originates from neural crest cells, exhibits enhanced cell migration by AQP1 under hypoxic conditions (24). Therefore, the AQP1 ion channel blocker AqB011 holds promise as a possible adjunct treatment to control neuroblastoma metastasis (23). The subcellular localization of AQP1 is controlled by phosphorylation via two different protein kinases (protein kinase C and PKA) and CaM may be implicated in the regulation of AQP1 (22). With the development of specific modulators, AQP1 could serve as a promising therapeutic target for selective intervention in neuroblastoma dissemination.

Angiogenesis, the formation of new blood vessels, plays a central role in tumor growth and metastasis. VEGF is the most potent inducer of angiogenesis (6). Several investigators have reported that inhibition of NF- κ B by nafamostat mesylate

suppressed VEGF expression (8,12). However, the present study demonstrated that nafamostat mesylate suppressed VEGF production from Neuro-2a cells without NF- κ B inhibition. Transcriptional factors, such as hypoxia-inducible factor 1, truncated glioma-associated oncogene homolog 1, and signal transducers and activators of transcription 3 reportedly regulate VEGF transcription and enhance expression VEGF (25-28). Therefore, these transcription factors in addition to NF- κ B will be assessed in future studies by the authors. Angiogenesis inhibition by nafamostat mesylate must be validated in humans by using humanized self-organized models, organoids, 3D cultures and human microvessel-on-a-chip platforms, especially those that are suitable for advanced imaging, such as transmission electron microscopy and expansion microscopy, because they enable real-time monitoring of mediators (29,30).

Although highly aggressive and invasive neuroblastoma remains an incurable disease, novel therapeutics could open up new possibilities to reduce mortality. High-throughput screening and computer-aided drug design should be applied for screening novel therapeutics because they can provide a unique insight that can support target validation in future studies (31,32).

The limitation of our study is the relatively small number of experiments. Thus, the findings of the present study need to be further validated in more extensive studies before clinical use. Only one murine neuroblastoma cell line, Neuro-2a, was employed in the present study. Given that nafamostat mesylate inhibits tumor cell proliferation with cell-type specificity (11), the anticancer effect of nafamostat mesylate may be different for different cell lines. Therefore, the anticancer effect should be assessed in human neuroblastoma cell lines before clinical application. Nevertheless, nafamostat mesylate is already in clinical use and has minimal adverse effects than the usual cytotoxic anticancer agents used for neuroblastoma (11). Thus, it is concluded that nafamostat mesylate could be a useful therapeutic modality for treating neuroblastoma.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

MM, HT, and MH conceived and designed the study. MM and HT performed the experiments and organized all the data. MM, HT, KN, RH, TO, DN, KA, SI, and MH analyzed and interpreted the data as well as reviewed and edited the manuscript. HT wrote the paper. MM, HT, and MH confirm the authenticity of all the raw data. All authors read and approved the manuscript and agree to be accountable for all aspects of

the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All animal procedures and experiments were performed according to a protocol approved by the Animal Ethics Committee (Permission number 27-34), Mie University Graduate School of Medicine, Tsu, Mie, Japan.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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