

● REVIEW

Nafamostat mesylate attenuates the pathophysiologic sequelae of neurovascular ischemia

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Abstract

Nafamostat mesylate, an apparent *soi-disant* panacea of sorts, is widely used to anticoagulate patients undergoing hemodialysis or cardiopulmonary bypass, mitigate the inflammatory response in patients diagnosed with acute pancreatitis, and reverse the coagulopathy of patients experiencing the commonly preterminal disseminated intravascular coagulation in the Far East. The serine protease inhibitor nafamostat mesylate exhibits significant neuroprotective effects in the setting of neurovascular ischemia. Nafamostat mesylate generates neuroprotective effects by attenuating the enzymatic activity of serine proteases, neuroinflammatory signaling cascades, and the endoplasmic reticulum stress responses, down-regulating excitotoxic transient receptor membrane channel subfamily 7 cationic currents, modulating the activity of intracellular signal transduction pathways, and supporting neuronal survival (brain-derived neurotrophic factor/TrkB/ERK1/2/CREB, nuclear factor kappa B. The effects collectively reduce neuronal necrosis and apoptosis and prevent ischemia mediated disruption of blood-brain barrier microarchitecture. Investigational clinical applications of these compounds may mitigate ischemic reperfusion injury in patients undergoing cardiac, hepatic, renal, or intestinal transplant, preventing allograft rejection, and treating solid organ malignancies. Neuroprotective effects mediated by nafamostat mesylate support the wise conduct of randomized prospective controlled trials in Western countries to evaluate the clinical utility of this compound.

Key Words: *apoptosis; cerebrovascular; excitotoxicity; infarction; ischemia; nafamostat mesylate; necrosis; neuroprotection; serine protease; subarachnoid hemorrhage*

Introduction

Nafamostat mesylate and argatroban are serine protease inhibitors (Ozawa et al., 2003; Liu et al., 2017) which ameliorate neural injury in the setting of ischemic and hemorrhagic cerebral infarction (Figures 1 and 2; Chen et al., 2014; Kwon et al., 2015; Liu et al., 2017) and vasospasm ensuing from aneurysmal subarachnoid hemorrhage (Yanamoto et al., 1992, 1994a, b; Zhang et al., 2001; Ghali et al., 2018) in preclinical animal models (Table 1). Nafamostat mesylate was originally developed in Japan in the 1980's and marketed by Japan Tobacco in 1986 to treat acute pancreatitis (Ozawa et al., 2003; Park et al., 2011, 2020; Hirota et al., 2020) and disseminated intravascular coagulation (Ozawa et al., 2003; Minakata et al., 2019; Hirota et al., 2020;). These agents are now commonly used to anticoagulate patients with severely hypoglomerular chronic kidney injury during hemodialysis (Katase et al., 2006; Inoue et al., 2013; Doi et al., 2018) and cardiopulmonary bypass (Murkin, 1993; Yamamoto et al., 2007; Kikura et al., 2012; Okada et al., 2013; Yasuda et al., 2013; Sakamoto et al., 2014) in the Far East. The inspiring and promising findings of preclinical (Hagiwara et al., 2007; Schwertz et al., 2008; Li et al., 2009; Miyagi et al., 2009; Furukawa et al., 2010; Altshuler et al., 2012; Gobbetti et al., 2012) and clinical (Chen et al., 2019a) investigations continue to broaden the spectrum of diseases which may be effectively treated using these drugs. In this regard, authors have extensively investigated and provided robust evidence supporting

safety and efficacy of using these compounds to treat and mitigate thromboembolic, occlusive, ischemia/reperfusion injury following myocardial, intestinal, hepatic, and renal transplantation, systemic inflammatory response syndrome and sepsis, hyperacute, acute, and chronic rejection, graft-versus-host disease, and solid organ malignancies (Table 2) (Chen et al., 2019a). These medications may be administered enterally or parenterally, exhibit first order pharmacokinetics, potently inhibit the activity of several serine proteases, are metabolized via oxidation in the blood or via phase I liver reactions, and undergo rapid renal clearance (Yang et al., 2009; Chen et al., 2019a).

The internal carotid and vertebral arteries and leptomeningeal anastomoses between the external and internal carotid arteries provide the blood supply perfusing the cerebrum (Rengachary and Ellenbogen, 2005; Spetzler et al., 2015). Typical cerebral blood flow approximates 55 mL per 100 mg neural tissue per minute; a decrease of the cerebral blood flow below 20 mL/100 g/min severely compromises the activity of neurolemmal membrane ion pumps and active transporters, contributing to osmotic cell swelling, though biomolecular processes are sufficiently preserved to eschew cellular necrosis or apoptosis (Rengachary and Ellenbogen, 2005; Spetzler et al., 2015). These effects are initially well tolerated permitting therapeutic intervention to prevent cell lysis (Rengachary and Ellenbogen, 2005; Spetzler et al., 2015). Neurons are incapable of sustaining normal biometabolic processes and

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doi: 10.4103/1673-5374.284981

Received: November 27, 2019

Peer review started: December 7, 2019

Accepted: March 27, 2020

Published online: June 19, 2020

Table 1 Effects of nafamostat mesylate

Reduces cerebral infarct volume
Reduces cerebral edema
Reduces thrombin generation and activation
Reduces blood-brain barrier disruption
Reduces PKCa activation of RhoA Rho-kinase
Reduces myosin light chain 2 phosphorylation
Reduces ischemia mediated downregulation of ZO-1 and occludin
Reduces infiltration of leukocytes
Decreases macrophage chemoattractant protein 1
Reduces inflammatory cytokines, IL-1 β , TNF- α
Upregulates anti-inflammatory cytokines, IL-4, IL-10, CD206, TGF- β
Downregulates TRPM7 currents
Upregulates BDNF/TrkB/ERK1/2/CREB
Downregulates NF- κ B activity
Upregulates Akt/eNOS
Upregulates glycogen synthase kinase 3
Downregulate apoptotic signaling
Decreases PAR-2 activation
Decreases ANGPT2 activation
Prevents IFN-g mediated upregulation of PD-L1
Potentiates antitumor CD8 cytotoxic T cells
Potentiates granzyme perforin mechanism of CD8 cytotoxic T cells
Prevents cathepsin B cleavage of Ebola virus cell surface glycoprotein
Prevents infection with Middle East coronavirus infection
Prevents chlamydial arthritic inflammation
Downregulates KRAS, BRAF, PIK3CA signaling
Downregulates p50/p65 nuclear transcriptional activity
Upregulates p53 and RELB

Akt: Protein kinase B; ANGPT2: angiopoietin 2; BDNF: brain-derived neurotrophic factor; BRAF: B-Raf and v-Raf murine sarcoma viral oncogene homolog B; CREB: cAMP response element binding protein; eNOS: endothelial nitric oxide synthase; ERK1/2: extracellular signal-regulated protein kinases 1 and 2; IFN- γ : interferon gamma; IL: interleukin; KRAS: Kirsten ras oncogene; NF- κ B: nuclear factor kappa B; PAR-2: protease activated receptor 2; PD-L1: programmed death ligand 1; PIK3CA: phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; RELB: avian reticuloendotheliosis viral oncogene homolog B; TGF- β : transforming growth factor-beta; TNF- α : tumor necrosis factor-alpha; TrkB: tropomyosin receptor kinase B; ZO-1: zonula occludens 1.

surviving should the cerebral blood flow decrease, and be maintained, below 8 mL/100 g/min greater than a few minutes. In this setting, the neurons rapidly succumb to necrosis and apoptosis (Rengachary and Ellenbogen, 2005; Spetzler et al., 2015). Rapidly dropping the cerebral metabolic rate of oxygen consumption through hypothermia or supratherapeutic doses of barbiturates or reestablishing normal cerebral perfusion represent the chief neural salvific and protective strategies in the presence of severe and sustained cerebral ischemia and consequent neural tissue hypoxia (Rengachary and Ellenbogen, 2005; Spetzler et al., 2015).

Several available neuroprotectants (e.g., lazaroids, nimodipine) reduce neuronal metabolic consumption of oxygen and glucose sufficiently to protect the cerebrum in cases of profound cerebral ischemia (Cahill and Hall, 2017; Ehrlich et al., 2019). Nafamostat mesylate and gabexate mesylate are serine protease inhibitors with an extraordinarily broad range of molecular biologic effects (Liu et al., 2017; Chen et al., 2019a), which have found remarkable promise

Table 2 Clinical and investigational indications of nafamostat mesylate

Ischemic cerebral infarct
Hemorrhagic cerebral infarct
Subarachnoid hemorrhage
Hemodialysis anticoagulation
Cardiopulmonary bypass anticoagulation
Acute pancreatitis
Post-endoscopic retrograde cholangiopancreatography pancreatitis prophylaxis
Disseminated intravascular coagulation
Systemic inflammatory response syndrome
Septic shock
Organ ischemia reperfusion injury, thromboembolic and transplant-related
Viral infections
Graft versus host disease
Alloimmune transplant reactions
Arthritic inflammation
Reactive airway disease
Pancreatic adenocarcinoma
Colonic adenocarcinoma
Multiple myeloma
Cardiovascular diseases

in ameliorating the extent and severity of injury to neural tissues in the setting of ischemia to the cerebrum ensuant from neurovascular embolic occlusion and intraparenchymal hemorrhage (**Figures 1 and 2**; Chen et al., 2014; Kwon et al., 2015; Liu et al., 2017) and vasospasm in the setting of subarachnoid hemorrhage (Yanamoto et al., 1992, 1994a, b; Zhang et al., 2001; Ghali et al., 2018) according to preclinical animal models and clinical trials conducted in Japan, China, and Korea. These studies have consistently demonstrated evidently remarkable reductions of cerebral infarct volume and cerebral edema, rescue of neuronal and synaptic microarchitecture, and upregulation of nerve growth factors in the setting of neurovascular ischemia (**Figures 1–6**; Chen et al., 2014; Kwon et al., 2015; Liu et al., 2017). These results collectively indicate and provide evidence supporting the utility of further evaluating the neuroprotective effects of these compounds in prospective randomized controlled trials in western countries and throughout the developed countries (Chen et al., 2019a, b). It is thus our principal goal and aim to discourse upon the mechanisms underlying nafamostat mesylate mediated mitigation of neurodegenerative sequelae ensuing from compromise of the blood supply providing oxygen, glucose, and metabolic nutrients to the neurons and glia (Liu et al., 2017).

Database Search Strategy

We performed an electronic search of the PubMed database with keywords and MeSH terms including “nafamostat mesylate,” “nafamostat,” “mesylate,” “FUT-175,” “serine protease inhibitor,” “serine protease inhibitors,” “cerebrovascular,” “neurovascular,” “cerebral,” “ischemia,” “infarction,” “subarachnoid hemorrhage,” “intracranial hemorrhage,”

“necrosis,” “apoptosis,” “neuroprotection,” “molecular,” AND “mechanisms.” We identified relevant studies from 1981 through 2020. Articles were evaluated by Prof. G. Z. Ghali and Prof. M. G. Z. Ghali to identify the extent of neuroprotection conferred by administration of nafamostat mesylate in animal models of ischemic cerebral infarction, intracranial hemorrhage, and subarachnoid hemorrhage and molecular mechanisms contributing to the neuroprotection.

Early Experience

Since the initial development of nafamostat mesylate in the 1980's and marketing in 1986 by Japan Tobacco to treat acute pancreatitis (Fujii and Hitomi, 1981; Hirota et al., 2020) and disseminated intravascular coagulation (Ozawa et al., 2003), its use was later extended to anticoagulating patients undergoing hemodialysis (Katase et al., 2006; Inoue et al., 2013; Choi et al., 2015) or cardiopulmonary bypass (Murkin, 1993; Sakamoto et al., 2014) and its preclinical and clinical investigational uses subsequently broadened to the treatment of systemic inflammatory response syndrome and sepsis, mitigation of thromboembolic, occlusive, and transplant related organ ischemia, graft versus host disease, and solid organ malignancy (Chen et al., 2019a). Nafamostat mesylate was shown to effectively reduce mortality in experimental acute pancreatitis in rats when delivered at infusion doses of 0.5 to 50 mg/kg/min (Iwaki et al., 1986). Several authors would later provide evidence supporting efficacy of nafamostat mesylate in preventing the development of iatrogenic pancreatitis following endoscopic retrograde cholangiopancreatographic interrogation of the biliary and pancreatic ducts and small bowel (Yuhara et al., 2014; Minakata et al., 2019).

Early investigators evaluated and contrasted the utility of continuous intravenous infusion versus regional arterial infusion of these agents (Iwaki et al., 1986). In early clinical trials evaluating the clinical utility of nafamostat mesylate in anticoagulating patients (Minakata et al., 2019), the compounds were variably dosed and compared against unfractionated heparin. The clinical utility of nafamostat mesylate has extended to anticoagulating patients undergoing life-saving extracorporeal membrane oxygenation (Han et al., 2019a), though characteristically generates higher rates of bleeding compared with unfractionated heparin (16.4% vs. 7.1%; Lim et al., 2016). In contrast, patients undergoing hemodialysis anticoagulated with unfractionated heparin experience higher rates of bleeding (16% vs. 6.6%) compared with those anticoagulated with nafamostat mesylate (Makino et al., 2016). Doses of nafamostat mesylate ranging from 0.2 to 0.5 mg/kg generated effective anticoagulation in patients undergoing hemodialysis (Park et al., 2015). Use of nafamostat mesylate in the treatment of individuals developing disseminated intravascular coagulation consequent to a broad spectrum of etiologies at doses ranging from 0.06–0.2 mg/kg per day effectively yielded clinical resolution rates of 40.3% and 56.3% on days 7 and 14 respectively. Accumulative evidence in preclinical animal models and clinical trials continued to expand a firm evidence base supporting potent efficacy and exceptional safety of nafamostat mesylate in the

treatment of acute enzymatic inflammation of patients with pancreas. Uwagawa et al. (2009a, b, 2013) evaluated the efficacy and safety of the combination therapy using nafamostat mesylate and gemcitabine in the treatment of pancreatic adenocarcinoma (1000 mg/m² body surface area). Nafamostat mesylate was administered on days 1, 8, and 15 of a 28-day therapy at a dose of 4.8 mg/kg, starting at 2.4 mg/kg and increasing in increments of 1.2 mg/kg, starting 24 hours prior to administration of gemcitabine therapy (Uwagawa et al., 2009a, b, 2013). The combinatorial therapeutic modality effectively and promisingly demonstrated greater efficacy compared with gemcitabine monotherapy (Uwagawa et al., 2009a, b, 2013). Lack of adverse effects at any of the utilized doses inspires the conduct of clinical trials to further evaluate the utility of this set of compounds in the treatment of carcinomas and sarcomas (Uwagawa et al., 2009a, b, 2013).

The serine protease inhibition and attenuation of innate and adaptive immune mechanisms mediated by nafamostat mesylate naturally generates a set of hypotheses indicating putative utility in preventing virion host cell fusion. Authors have accordingly sought to exploit these properties to treat individuals infected with the Ebola flavivirus (Nishimura and Yamaya, 2015), a highly contagious hemorrhagic fever virus generating a clinical syndrome bearing striking resemblance to that caused by Marburg and Lassa fever viruses (~25–60%), with comparatively greater mortality rates in untreated patients (~90%). Patients infected with these single stranded ribonucleic acid (RNA) viruses characteristically develop and suffer from profoundly severe gastrointestinal symptomatology, characterized by a prodrome of severe nausea and vomiting and profuse diarrhea, preceding widespread compromise of organ function and disseminated intravascular coagulation. Mechanistically, treatment with nafamostat mesylate effectively prevented endosomal cathepsin B mediated processing of Ebola cell surface glycoprotein, a necessary and critical step in the progression and potentiation of virion fusion with cell membranes (Nishimura and Yamaya, 2015). Yamamoto et al. (2016) demonstrated use of this agent also effectively prevents the establishment of infection with Middle East respiratory syndrome coronavirus (Yamamoto et al., 2016). A recent report also provides evidence indicating utility of using nafamostat mesylate to treat patients infected with dengue hemorrhagic fever virus (Rathore et al., 2019).

Use of nafamostat mesylate may generate a broad spectrum of adverse effects ranging in clinical severity (**Table 3**) (Furukawa et al., 2010; Uwagawa et al., 2013; Kim et al., 2016; Sawada et al., 2016; Minakata et al., 2019; Shindo et al., 2019). Approximately 0.9% of patients with leukapheresis receiving this medication may experience headache, nausea, and fever (Sawada et al., 2016). Abnormalities of the serum chemistry profile in individuals administered this agent in the setting of hemodialysis may occur consequent to effects of the drug and include hyperkalemia (Furukawa et al., 2010) and hyponatremia (Furukawa et al., 2010). Serine protease inhibition by nafamostat mesylate may compromise the hepatocyte bio-organic processes sufficiently to generate clinically and

Table 3 Adverse effects of nafamostat mesylate

Headache
Nausea
Fever
Hyponatremia
Hyperkalemia
Hepatic dysfunction
Anaphylaxis
Myelosuppression
Disseminated intravascular coagulation
Bleeding

biochemically evident hepatic dysfunction (Furukawa et al., 2010; Haruki et al., 2013a). Nafamostat mesylate mediated attenuation of pathways promoting cellular proliferation (e.g., nuclear factor kappa B [NF- κ B]) may sufficiently compromise myelopoiesis to cause anemia, leukopenia, and/or thrombocytopenia and derivative clinical sequelae, experienced by a small fraction of patients receiving this medication to treat pancreatic adenocarcinoma (Uwagawa et al., 2009a, b, 2013). IgE-induced mast cell degranulation mediated anaphylactic reactions represent the most widely observed and reported life-threatening adverse effect (Kim et al., 2016; Shindo et al., 2019). Nafamostat mesylate may paradoxically and coordinately ameliorate disseminated intravascular coagulation and precipitate the condition in patients harboring a predisposing comorbidity though without underlying coagulopathy (Minakata et al., 2019). Accordingly, extension of serine protease inhibition mediated by nafamostat mesylate to antagonizing the proteolytic enzymatic activities of clotting factors of the extrinsic and intrinsic cascades, anticlotting (i.e., antithrombin III, protein C, and protein S), fibrinolytic (i.e., plasmin, tissue-plasminogen activator), and antifibrinolytic (i.e., plasminogen activator inhibitor-1) factors would expectedly and preferentially halt the progression of established disseminated intravascular coagulation. In patients not suffering from underlying coagulopathy, this set of effects may sufficiently shift the delicate balance among and between these pathways to generate the onset of coagulopathy.

The Role of Serine Proteases in the Pathophysiology of Ischemic Cerebral Infarction

Serine proteases contribute prominently to neuronal necrosis and apoptosis in the setting of neuronal ischemia. In brief, endothelial denudation mediated activation of the extrinsic clotting cascade by exposed tissue factor and high molecular weight kininogen/kallikrein/factor XII mediated activation of the intrinsic clotting cascade converges upon proteolytically generating activated factor Xa from factor X. Factor Xa proteolytically cleaves prothrombin into thrombin in the presence of factor Va, phospholipids, and calcium, liberating the enzymatic activity of this serine protease to cleave fibrinogen cross-linking platelets, adhered to the endothelium via interactions of platelet surface GpIb receptor with von Willebrand

factor, via glycoprotein IIb/IIIa into fibrin, strengthening the interaction, and factor XIII into factor XIIIa, stabilizing the platelet-fibrin thrombus. Thrombin mediated proteolytic cleavage of factors V into activated factor Va, a cofactor of activated factor Xa, and factor VIII into activated factor VIIIa, a cofactor of factor IXa generates their activated oligopeptide. Thrombin coordinately negatively modulates the coagulation cascade by potentiating the actions of thrombomodulin (Davie et al., 1991; Grand et al., 1996).

In vitro studies have provided evidence indicating neurons express post-transcriptionally modified messenger ribonucleic acid transcripts of the serine proteases prothrombin (Weinstein et al., 1995; Krenzlin et al., 2016) and factor X (Shikamoto and Morita, 1999). Interruption of the blood flow and consequent ischemia to the cerebrum markedly enhance plasma levels and tissue activity of the serine proteases thrombin, plasmin, and kallikrein (Girolami et al., 2020). Ischemia induced compromise of blood-brain barrier microarchitectural integrity (Thevenet et al., 2009; Chen et al., 2012; Gob et al., 2015) may effectively enhance the transmigration of serine proteases from the circulation into privileged nerve tissues (Xi et al., 2003; Chen et al., 2014; Ben Shimon et al., 2020). Burgeoning evidence continues to demonstrate that the enzymatic catalytic activity of serine proteases synergistically enhances glutamate excitotoxicity and processes mediating necrotic and apoptotic neuronal degeneration in the setting of a variety of neurovascular neuropathologic cascades, including ischemic cerebral infarction (Wu et al., 2010; Yoon et al., 2013; Chen et al., 2014; Gob et al., 2015; Stein et al., 2015; Liu et al., 2017; Yang et al., 2017; Ghali et al., 2018), intracerebral hemorrhage (Ghali et al., 2018), subarachnoid hemorrhage (Ghali et al., 2018), and traumatic brain injury (Festoff and Citron, 2019), though authors have recently indicated that endogenously generated thrombin may mediate neuroprotective effects through the activation of astrocytes (Rajput et al., 2020). Nafamostat mesylate may blunt serine proteases directly by reducing enzyme catalytic activity and by attenuating ischemia mediated accentuation of serine protease activity and upregulation of expression (Figure 3) (Chen et al., 2014). Nafamostat mesylate was specifically shown to reduce ischemia mediated augmentation of thrombin expression in striatum ipsilateral to experimental MCA territorial ischemia (Chen et al., 2014).

Thrombin may severely perturb cellular processes and may initiate and/or exacerbate a spectrum of neurodegenerative processes through the activation of plasma membrane platelet activating receptors (PARs; Xi et al., 2003), of which there exist multiple subtypes. Thrombin preferentially activates PAR-1, 3, and 4 and trypsin preferentially activates PAR-2. PAR-1 represents the most widely expressed subtype across neuronal and glial membranes (Dihanich et al., 1991; Sokolova and Reiser, 2008) and undergoes marked upregulation in response to anoxia (de Castro Ribeiro et al., 2006). Thrombin mediated activation of the NOD-like receptor and pyrin domain-containing 3 (NLRP3) inflammasome in macrophages (Rossol et al., 2012) and promotion of microglial activation (Lee et al., 2005) widen the spectrum of the

mechanisms by which this monomeric serine protease mediates neurodegenerative effects, and reciprocally, the mechanisms by which nafamostat mesylate and argatroban may protect the cerebrum in the condition of ischemia. Studies demonstrating intracerebroventricular administration of thrombin precipitates cognitive deficits in rats provide direct evidence supporting serine proteases contributing to the development of neuronal cytotoxicity (Chen et al., 2012).

Nafamostat Mesylate Mediated Inhibition of Serine Proteases Prevents Penumbra Infarct Transformation of Ischemic Cerebrum

Nafamostat mesylate was incidentally found to reduce the risk of perioperative ischemic stroke (0.5% vs. 1.5%) when administered to patients undergoing cardiopulmonary bypass to treat heparin resistance (Kikura et al., 2012). The protocol provided patients with bolus (10–20 mg) and maintenance (25–50 mg/h) doses of nafamostat mesylate and a continuous maintenance heparin infusion delivered at 100 U/kg per hour. Several authors have provided evidence indicating that nafamostat mesylate profoundly reduces infarct volume and neuronal degeneration in animals subjected to experimental middle cerebral artery territorial ischemia (**Figure 3**) (Chen et al., 2014; Kwon et al., 2015; Wang et al., 2016; Liu et al., 2017). Liu et al. (2017) conducted a set of studies providing us with experimental data specifically evaluating the gross neuroanatomic, microanatomic, biomolecular, and neurofunctional effects of serine protease inhibition using nafamostat mesylate and argatroban on middle cerebral artery territorial ischemia. The authors demonstrated profound dose-dependent reductions of cerebral infarct volume and extent of edema and preservation of synaptodendritic eloquence and complexity in animals treated with these compounds (Liu et al., 2017).

The serine protease inhibitors appear to attenuate nerve injury in cerebral ischemia principally by limiting penumbra infarction (Liu et al., 2017). Among the disparate and pleiotropic cellular and biochemical effects mediated by nafamostat mesylate, we accordingly propose the inhibition of the enzymatic catalytic activity of serine proteases, especially inhibition of the enzymatic catalytic activity of thrombin may contribute most prominently to mitigating ischemia induced neuronal degeneration mediated by these agents (Chen et al., 2019a, b). A myriad of biomolecular pathways, some precisely interrogated and dissected and others yet to be thoroughly elucidated, also mechanistically contribute to the neuroprotective effects mediated by these drugs (Chen et al., 2014; Kwon et al., 2015; Liu et al., 2017). Accordingly, nafamostat mesylate modulates the activity and behavior of several intracellular signal transduction pathways promoting cellular and neuronal survival and proliferation, including brain-derived neurotrophic factor (BDNF)/tyrosine kinase B (TrkB)/extracellular regulated kinase 1/2 (ERK1/2)/cyclic AMP response element binding protein (CREB) and NF- κ B (Liu et al., 2017). Serine protease inhibitors attenuate and reduce the elaboration of neuroinflammatory cytokines, the

activation and chemotaxis of microglia, ischemia-induced distortion of blood-brain barrier microarchitecture (Wang et al., 2016), and the endoplasmic reticulum stress response (Kwon et al., 2015) to ischemia and modulate potentially excitotoxic divalent and monovalent cationic currents (Chen et al., 2010b, c). The panoply and conglomerate of neuroprotective effects mediated by these agents elucidated and substantiated *in vitro* and in animal models *in vivo* and the extensive evidence base revealing legion and myriad mechanisms contributing to the described mitigation and attenuation of neuronal and astroglial injury indicates incredible promise in the use of nafamostat mesylate to profoundly reduce neurodegeneration in patients developing neurovascular territorial ischemia (Chen et al., 2014; Kwon et al., 2015; Liu et al., 2017).

Nafamostat Mesylate Attenuates Nerve Injury and the Effects of Neurovascular Ischemia

Treatment with nafamostat mesylate and argatroban mitigates nerve injury in experimental ischemic (Chen et al., 2014) and hemorrhagic (Phongsisay et al., 2008; Li et al., 2009; Nakamura et al., 2010) cerebral infarction in preclinical animal models. Use of these serine protease inhibitors was demonstrated to powerfully attenuate cerebral infarct volume ipsilateral to middle cerebral artery territorial ischemia and enhance neuromotor and cognitive scores in rat models of ischemic stroke and reduce brain edema and deoxyribonucleic acid injury in rat models of intracerebral hemorrhage (Chen et al., 2014; Kwon et al., 2015; Liu et al., 2017). Serine protease inhibition of clotting factors by nafamostat mesylate and argatroban may in theory enhance the risk of hemorrhagic transformation of an ischemic infarct or potentiate bleeding in intracerebral hemorrhage, though hematoma volume was not enhanced by treatment with nafamostat mesylate in collagenase-treated rats (Nakamura et al., 2010). This provides evidence indicating that nafamostat mesylate may be safely used to mitigate the sequelae of neuronal ischemic injury in patients with intracranial hemorrhage without exacerbation of the initial bleeding event. The anticoagulant properties of nafamostat mesylate may thus obviate the use of enoxaparin, unfractionated heparin, or fondaparinux thromboprophylaxis in patients having sustained neurovascular injury. Treatment with nafamostat mesylate consistently attenuates cerebral vasospasm in animal models (Yanamoto et al., 1992, 1994a, b; Murkin, 1993; Zhang et al., 2001; Ghali et al., 2018) and clinical trials (Yamamoto et al., 1992b; Kaminogo et al., 1998; Ghali et al., 2018) of subarachnoid hemorrhage. Accordingly, contact of the serine protease thrombin and platelet-derived growth factor leads to vasoconstrictor vasospasm and vascular smooth muscle cell hyperplasia and hypertrophy consequent to the release of arterial blood under high pressure irritating the abluminal surface of arteries of the circle of Willis coursing within the subarachnoid cisterns and proximal anterior and middle cerebral arteries (Ghali et al., 2018). Pharmacologically attenuating these pathways using serine protease

inhibitors could therapeutically mitigate subarachnoid hemorrhage related vasospasm (Yanamoto et al., 1992, 1994a, b; Kaminogo et al., 1998; Zhang et al., 2001; Llull et al., 2017). Further studies are necessary to more precisely determine the effects of nafamostat mesylate, gabexate mesylate, and argatroban on cerebral microcirculatory physiology, cerebral vasomotion, and delayed cerebral ischemia. Findings generated by these studies will accordingly more fully illumine our understanding of the mechanisms underlying nafamostat mesylate mediated attenuation of nerve injury.

Mechanisms underlying Nafamostat Mesylate-Mediated Attenuation of Nerve Injury following Ischemic Stroke

Nafamostat mesylate and argatroban exert potent neuroprotective effects mediated via several biochemical pathways. These mechanisms include inhibition of serine protease catalytic activity, attenuation of neuroinflammatory cascades, and modulation of biomolecular signal transduction networks (Chen et al., 2014; Kwon et al., 2015; Liu et al., 2017). Authors have made significant strides into elucidating the mechanistic underpinnings of these pathways through the elegant conduct of thoughtfully designed investigations in preclinical animal models of ischemic cerebral infarction, intracranial hemorrhage, and subarachnoid hemorrhage (Chen et al., 2014; Kwon et al., 2015; Liu et al., 2017; Ghali et al., 2018). There seems to be a general consensus on the neuroprotective effects of nafamostat mesylate through attenuating the enzymatic activity of thrombin and that of other serine proteases. In putatively commensurate neuroprotective measure, nafamostat mesylate potently attenuates the elaboration of neuroinflammatory cytokines by the effector cells of the immune system, reduces the extent of leukocyte chemotaxis to sites of ischemic injury and the blunt activation of microglia (Li et al., 2016), and effectively mitigate ischemia induced distortion of blood-brain barrier microarchitecture (Wang et al., 2016). Nafamostat mesylate mediated modulation of the BDNF/TrkB/ERK1/2/NF- κ B pathway (Liu et al., 2017) and attenuation of the endoplasmic reticulum stress response (Kwon et al., 2015) also coordinately promote neuronal survival and prevent neurodegeneration. These mechanisms, in confluence, promote neuronal salvage, enhance resilience to oxygen and glucose deprivation (Wang et al., 2016), and effectively dampen the apoptotic response to neuronal ischemia (Kwon et al., 2015). Transient receptor membrane channel subfamily 7 (TRPM7) current and calcium flux modulation by these serine protease inhibitors may prevent the neurodegenerative sequelae of ischemia by attenuating excitotoxic cationic currents. Treatment with nafamostat mesylate downregulates the expression of matrix metalloproteinase-9 *in vitro* (Fujiwara et al., 2011b), which reduces the degradation of extracellular matrix. We accordingly discuss these pathways in order to provide mechanistic insight into the neuroprotective effects exerted by these compounds.

Nafamostat Mesylate Rescues Blood-Brain Barrier Microarchitecture in Neurovascular Ischemia

Tight junction complexes coupling endothelial cells, the abluminal basement membrane, astrocytic foot processes, and pericytes collectively constitute a formidable blood-brain barrier, preventing the entry of large and powerfully charged serum macromolecules and ions into the neural interstitium (Chen et al., 2019b; Tjakra et al., 2020). A variety of mechanisms synergistically converge to disrupt the delicate blood-brain barrier microarchitecture in neurovascular ischemia (Kunze and Marti, 2019; Li et al., 2019). Thrombin and serine proteases potentially enhance neuroendothelial permeability (Holinstat et al., 2003; Hun Lee et al., 2015) by compromising the integrity of interendothelial cell apical tight junctions through activation of PAR-1 (Rajput et al., 2014). Thrombin mediated activation of PAR-1 upregulates the activity of PKC α (Gingrich and Traynelis, 2000; Sandoval et al., 2001; Xi et al., 2003; Chen et al., 2010a; Woitzik et al., 2011; López et al., 2019). PKC α phosphorylatively activates RhoA/ROCK (Van Aelst and D'Souza-Schorey, 1997; Curry and Adamson, 2010; Fernández-López et al., 2012), which phosphorylatively activates myosin light chain 2 (MLC2) (Mehta and Malik, 2006; Koller et al., 2020), enhancing endothelial cellular contractility (Van Aelst and D'Souza Schorey, 1997; Singh et al., 2007; Saito et al., 2011) and intercellular porosity by expanding the paracellular routes. Treatment with nafamostat mesylate reduces endothelial permeability by attenuating ischemia mediated reductions of ZO-1 and occludin expression (Brown and Davis, 2002), tight junction proteins maintaining endothelial integrity (Michinaga et al., 2020; Namyen et al., 2020). Treatment with the serine protease inhibitors nafamostat mesylate and argatroban prevents ischemia mediated PKC translocation to membrane-enriched cellular compartments in the presence of thrombin and under conditions of oxygen-glucose deprivation (Mole-dina et al., 2001; Escolar et al., 2006; Wang et al., 2016). Small interfering ribonucleic acid and molecular antagonists of PKC α (Mehta et al., 2001; Holinstat et al., 2003; Alavian et al., 2012; Wang et al., 2018) and RhoA/ROCK [via the inhibitor Y27632 (Essler et al., 1998; Gavard and Gutkind, 2008) or fasudil (Niego et al., 2017; Hamano et al., 2019; Ke et al., 2019)]. These mechanisms offer medicinal chemists and pharmacologists a molecular set of targets by which to therapeutically modify and rescue blood-brain barrier microarchitecture in neurovascular ischemia and subarachnoid hemorrhage (Andjelkovic et al., 2019; Li et al., 2020).

The use of pharmacologic inhibitors of thrombin and serine proteases may effectively attenuate neurovascular ischemia mediated blood-brain barrier distortion (Chen et al., 2010a; Woitzik et al., 2011; Li et al., 2015; Wang et al., 2016; Machida et al., 2017). Treatment with nafamostat mesylate was shown to effectively reduce blood-brain barrier disruption in a rat model of transient middle cerebral artery territorial ischemia *in vivo* and attenuate ischemia-mediated perturbation of tight junction expression and cytoskeletal

protein arrangement in an endothelial and astrocyte cell model of oxygen and glucose deprivation *in vitro* (Wang et al., 2016). We use the findings the study to validate a set of interpretations predicated upon nafamostat mesylate treatment mediated reductions of cerebral edema to be principally reflect correlated attenuations in blood-brain barrier distortion, though we ascribe correlated reductions of the cerebral infarct volume to more preferentially reflect serine protease inhibition and attenuation of neuroinflammatory processes observed *in vivo* (Wang et al., 2016).

Nafamostat Mesylate Rescues Synaptodendritic Eloquence and Microarchitecture and Nerve Growth Factors

BDNF (Berretta et al., 2014; Giacobbo et al., 2020; Zhang et al., 2020b), nerve growth factor (NGF) (Stepanyan et al., 2018; Gudasheva et al., 2019; Luan et al., 2019), and neurotrophin 3 (NT3) (Pasarica et al., 2005; Zhang et al., 2012; Chung et al., 2017) are neuronally derived neuronally active growth factors, the expression of which exhibit complex alterations in cerebral hemispheres ipsilateral and contralateral to embolic neurovascular ischemia, the upregulation of which correlates with neurological outcomes, and the administration of which exerts neuroprotective effects. The synthesis and elaboration of these proteins into neural interstitium powerfully promote neuronal growth and enhance synaptic neuroplasticity, which may thus critically contribute to neural regeneration following neurovascular ischemia (Zhang et al., 2012; Gudasheva et al., 2019). In this regard, Liu et al. (2017) provided experimental evidence at the cellular and biochemical levels indicating nafamostat mesylate and argatroban mediate potent neuroprotective effects. Treatment with these agents was shown to significantly reduce ischemia induced synaptodendritic pruning (Figure 4), dampen PSD-95 and synaptophysin expression (Figure 5), effectively rescue synaptodendritic eloquence, powerfully and differentially modify the expression patterns of NGFs in the cerebrum ipsilateral and contralateral to middle cerebral artery territorial ischemia, exhibiting locoregional heterogeneity, threshold properties, and dose dependent variance (Liu et al., 2017). The serine protease inhibitors nafamostat mesylate and argatroban were shown to amplify middle cerebral artery territorial ischemia mediated upregulation of growth factors (BDNF, NGF, and NT3) in sensorimotor cortex contralateral to middle cerebral artery occlusion and attenuate ischemia mediated reductions in the expression of these proteins in sensorimotor cortex and hippocampal formation ipsilateral to middle cerebral artery occlusion (Liu et al., 2017). Specifically, in sensorimotor cortex contralateral to middle cerebral artery territorial ischemia, treatment with nafamostat mesylate and argatroban enhanced the expression of BDNF, though treatment with only the highest dose of nafamostat mesylate (1.0 mg/kg) proved capable of further enhancing ischemia mediated increases of NGF and NT3 expression levels (Liu et al., 2017). Middle cerebral artery territorial ischemia mediated reductions of BDNF were

effectively prevented by treatment with the highest dose of nafamostat mesylate (1.0 mg/kg), though treatment with the standard solitary dose of argatroban had no effect (Liu et al., 2017). Middle cerebral artery territorial ischemia mediated reductions of NGF and NT3 expression in ipsilateral sensorimotor cortex were not significantly modified by treatment with argatroban and the highest doses of argatroban (Liu et al., 2017). In hippocampal formation ipsilateral to middle cerebral artery territorial ischemia, treatment with argatroban (3.4 mg/kg) and the highest doses of nafamostat mesylate (1.0 mg/kg) effectively reversed ischemia mediated reductions in BDNF and NGF expression, though treatment only with the latter effectively prevented ischemia mediated reductions of neurotrophin 3 expression. Inhibition of serine protease activity powerfully reduced the extent of corticobulbar and corticospinal fiber degeneration (Figure 6) and enhanced neurofunctional motor (Liu et al., 2017) and cognitive scores (Chen et al., 2014) in middle cerebral artery territorial ischemia (Liu et al., 2017). These effects exerted by the serine protease inhibitors may thus effectively mitigate neurovascular ischemia mediated compromise of the capacity for neural recovery amongst neuronal ensembles constituting neural network arrays and coordinately enhance contralateral compensatory mechanisms to subsume the functions of infarcted parenchyma (Liu et al., 2017).

Nafamostat Mesylate Attenuates Neuroinflammation

Serine proteases enhance neuroinflammation mediated neural injury occurring in neurovascular ischemia. Authors have consequently and reasonably proposed dampening neuroinflammatory cascades may effectively reduce neuronal degeneration arising consequent to compromise of cellular metabolic processes in response to ischemic injury (Lee et al., 2007; Naito et al., 2020). Accordingly, several well-designed studies interrogating the mechanistic underpinnings of the salvific effects of nafamostat mesylate upon oxygen deprived neural tissue have provided robust evidence indicating treatment with these agents generates powerful attenuations of neuroinflammatory cascades across several cell lines (Li et al., 2016; Duan et al., 2018). Studies have specifically demonstrated treatment with nafamostat mesylate powerfully attenuates neuroinflammatory cascades instigated by neurovascular ischemia *in vivo* and *in vitro*. Nafamostat mesylate exerts myriad effects upon innate, cellular adaptive, and humoral adaptive immunity. Treatment with this agent reduces the synthesis and release of the inflammatory cytokines interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), inducible nitric oxide synthase (iNOS), and cyclooxygenase 2 (COX-2) and upregulates the synthesis and release of the anti-inflammatory cytokines IL-4, IL-10, transforming growth factor- β , and CD206. Treatment with nafamostat mesylate decreases infiltration of ischemogenically compromised sites of neural injury by neutrophils, macrophages, and lymphocytes, a set of effects presumably mediated through the inhibition of vascular adhesion molecule and macrophage chemoattrac-

tant protein 1 synthesis (Li et al., 2016). Nafamostat mesylate coordinately blunts interferon- γ mediated tumor cell upregulation of the membrane surface protein programmed death ligand 1, effectively enhancing the cytotoxic T cell response to tumor cells and preventing tumor capacity to evade cellular adaptive immunity (Homma et al., 2018).

Treatment with nafamostat mesylate was shown to reduce lipopolysaccharide mediated enhancement of nitric oxide synthase activity in RAW264.7 murine macrophages (Noguchi et al., 2003), coordinately with reductions in the expression of NF- κ B and phosphorylation and degradation of its modulatory inhibitor I κ B α in human umbilical vein endothelial cells (Noguchi et al., 2003). Nafamostat mesylate effectively reduced the expression of the lipopolysaccharide mediated increases in the expression of nitric oxide synthase, the proinflammatory macrophage derived cytokines IL-6 and IL-8, and matrix metalloproteinases and lipopolysaccharide mediated apoptosis in human trophoblasts (Nakatsuka et al., 2000). Treatment with nafamostat mesylate attenuated the expression and elaboration of IL-1 β (Han et al., 2018), IL-5, IL-6, IL-13, and IL-17. Treatment with nafamostat mesylate reduces infiltration of nerve tissue by neutrophils, macrophages, and T cells, effects mediated via reduced expression of the chemotactic proteins macrophage chemoattract protein-1 and intercellular adhesion molecules (ICAM-1) and vascular cell adhesion molecule-1 (Li et al., 2016). Li et al. (2016) demonstrated nafamostat mesylate generates powerful reductions in the expression of the inflammatory cytokines TNF- α and IL-1 β , iNOS, and COX-2 in rats with middle cerebral artery territorial ischemia generated via transient occlusion. Nafamostat mesylate was shown to coordinately enhance the expression of the anti-inflammatory molecules CD206, transforming growth factor- β , IL-4, and IL-10 (Li et al., 2016). In rat microglia exposed to thrombin under oxygen-glucose deprivation condition, treatment with nafamostat mesylate effectively reduced the expression of inflammatory cytokines and enhanced the expression of anti-inflammatory cytokines (Li et al., 2016). The effects of nafamostat mesylate upon inflammatory signaling cascades within neural tissue appear to be principally mediated through the inhibition of NF- κ B-mediated signaling (Li et al., 2016).

Microglia may differentially polarize towards inflammatory and anti-inflammatory types (Li et al., 2016). NF- κ B pathway activation preferentially favors phenotypic switching of these cells towards the inflammatory type of microglia (Zhang et al., 2019; Ganbold et al., 2020). Upon activation, microglia develop extensive cytoplasmic processes and undergo a marked upregulation in the production and secretory elaboration of proinflammatory cytokines and chemical mediators mediating chemotaxis and enhancing the expression of intercellular adhesion molecules (Liu et al., 2019; Mee-Inta et al., 2019). These effects synergistically enhance the margination of leukocytes from the circulating pool and the capacity for inflammatory cellular diapedesis into the neuronal interstitium and thus contribute to amplifying subsequent inflammatory-mediated and inflammatory-aug-

mented neuronal cell loss and architectonic distortion in areas subjected to active ischemia (Li et al., 2016). Authors have previously provided evidence indicating a tendency of microglia to preferentially migrate towards perivascular regions in active neurovascular ischemia, navigating preferentially according to, and in direct variance with, concentration gradients of thrombin. Thrombin activates microglia through the activation of membrane platelet activating receptors (García et al., 2010) and facilitates microglial chemotaxis towards zones of nerve tissue ischemia (Charo and Taubman, 2004). Nafamostat mesylate-mediated attenuation of NF- κ B signaling preferentially polarizes microglial differentiation toward the anti-inflammatory type (Aslanidis et al., 2015; Ni et al., 2015). Nafamostat mesylate may also modulate NF- κ B mediated microglial activation by suppressing degradation of its inhibitory I κ B α and through p65 phosphorylation. Nafamostat mesylate may also effectively reduce neuroinflammatory facilitation of ischemia induced neurodegeneration (Ito et al., 2015) by downregulating thrombin mediated activation of the NLRP3 inflammasome (Rossol et al., 2012), effects demonstrated *in vivo* and *in vitro* (Li et al., 2016).

Nafamostat Mesylate Inhibits N-methyl-D-Aspartate Glutamate Receptor Mediating Signaling by Binding to the NR2B Subunit and Modulates TRPM7 Currents

The activity of several membrane channels mediating intracellularly directed divalent and monovalent cationic fluxes are subjected to modulation by treatment with the serine protease inhibitory nafamostat mesylate (Chen et al., 2010b, c). These effects collectively indicate nafamostat mesylate may generate its neuroprotective effects by mitigating glutamate excitotoxicity mediated neuronal necrosis and apoptosis in cerebral ischemia, hypoxia, or anoxia. Fuwa et al. (2019) provided evidence indicating nafamostat mesylate and its congener sepimostat mediate potent neuroprotective effects chiefly through N-methyl-D-aspartate (NMDA) glutamatergic antagonism of the NR2B subunit at the ifenprodil binding site. Treatment with nafamostat mesylate and sepimostat effectively prevented NMDA mediated excitotoxicity *in vitro* in rat cortical neurons and prevented retinal toxicity *in vivo* following intravitreal injection. The neuroprotective effects of nafamostat mesylate were significantly reduced *in vitro* and *in vivo* by treatment with spermidine, a potent modulator of the NMDA receptor NR2B subunit. These results collectively indicate nafamostat mesylate and sepimostat generate neuroprotective effects by blunting and inhibiting NMDA glutamatergic signaling via the NR2B subunit.

Treatment with nafamostat mesylate powerfully modulates TRPM7 channel conductances (Chen et al., 2010b). TRPM7 channels mediate intracellularly directed divalent cationic magnesium (Zou et al., 2019) and calcium (Faouzi et al., 2017; Sun et al., 2009) currents. TRPM7 currents contribute to neurosynaptic calcium rises generating synaptovesicular fusion with the presynaptic membrane and consequent

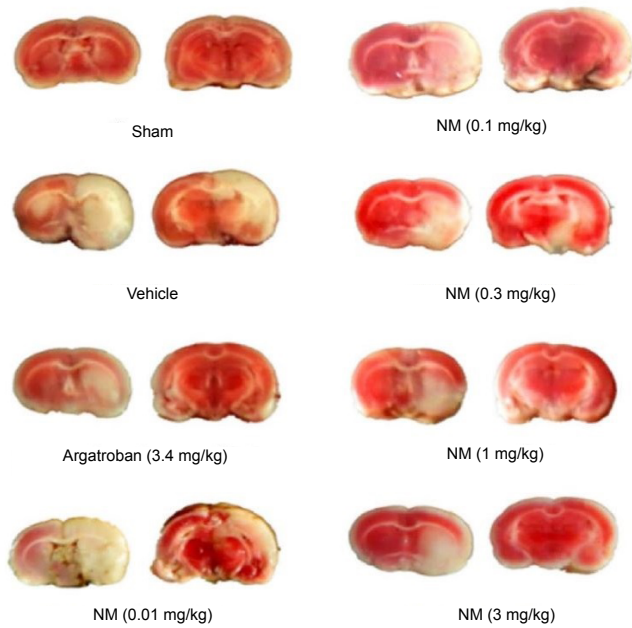


Figure 1 Serine protease inhibition decreases infarct volume in a middle cerebral artery rat model of cerebrovascular ischemia.

The neuroprotective effects of nafamostat mesylate (NM) on ischemic cerebrum exhibited dose-dependent efficacy. Untreated rats sustaining hemispheric ischemia via middle cerebral artery occlusion (vehicle) develop extensive territorial infarct. Normal cerebral parenchyma without cerebrovascular ischemia induced via MCAO is indicated for comparison (sham). Animal groups included a single dose of argatroban or varying doses of NM. Argatroban (3.4 mg/kg) and NM (0.1, 0.3, 1, and 3 mg/kg) significantly reduced infarct volume and cerebral edema. The 0.01 mg/kg dose of NM had no effect. The results collectively validate the threshold and dose dependence of the treatment with serine protease inhibitors to generate neuroprotective effects. Modified with permission from Figure 1 of Chen et al. (2014).

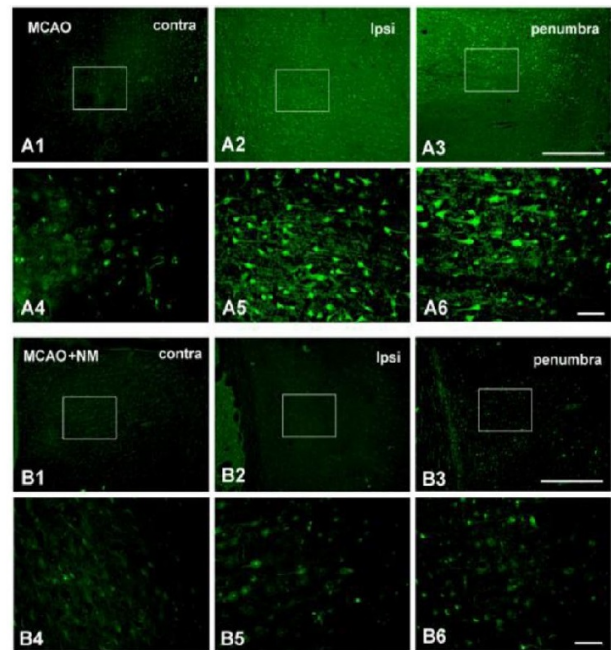


Figure 2 NM reduces ischemia induced neuronal degeneration.

Neuronal degeneration in animals having sustained territorial hemispheric ischemic infarction after middle cerebral artery occlusion without (A1–A6) versus with (B1–B6) NM neuroprotection. (A1, A4) Cerebral cortex contralateral (contra) to MCAO without NM neuroprotection. (A2, A5) Cerebral cortex ipsilateral (ipsi) to MCAO without NM neuroprotection. (A3, A6) Cerebral cortex in the penumbral region of MCAO without NM neuroprotection. (B1, B4) Cerebral cortex contralateral to MCAO with NM neuroprotection. (B2, B5) Cerebral cortex ipsilateral to MCAO with NM neuroprotection. (B3, B6) Cerebral cortex in the penumbral region of MCAO with NM neuroprotection. (A4–A6) Expansions of the boxes of A1–A3. (B4–B6) Expansions of boxes in B1–B3. Immunofluorescence of contralateral normal cortex and ipsilateral ischemic cortex and penumbral zones is demonstrated in untreated and treated animals. Middle cerebral artery territorial ischemia significantly increased the expression of the fluoro-Jade-B marker in ipsilateral cortex and penumbra, which was attenuated by treatment with the serine protease inhibitor NM. Modified with permission from Figure 2 of Kwon et al. (2015). MCAO: Middle cerebral artery occlusion; NM: nafamostat mesylate.

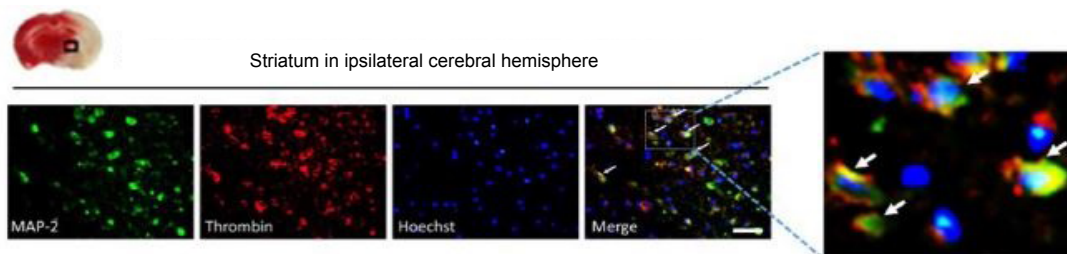


Figure 3 Nafamostat mesylate decreases middle cerebral artery occlusion induced striatal thrombin upregulation.

Middle cerebral artery territorial ischemia upregulates thrombin expression in the ipsilateral striatum. Treatment with the serine protease inhibitor nafamostat mesylate attenuates these increases. The rightmost subpanel represents an expansion of the subpanel in the subpanel labeled 'merge.' Merge image indicates neurons fluorescently double labeled with antibody against microtubule associated protein 2 and thrombin. Hoechst is a neuronal membrane permeant, minor groove binding blue colored fluorescent DNA stain labeling neurons. The white arrows indicate colabeling of thrombin with fluorescein-dextran or neurons. Merge image scale bar = 50 μ m. Modified with permission from Figure 2 of Chen et al. (2014).

elaboration of neurotransmitter into the synaptic cleft synergistically with membrane voltage gated calcium channels (Brauchi et al., 2008). Elevated levels of cytosolic calcium potentiate the enzymatic activity of intracellular phospholipases, proteases, and nucleases mediating necrotic neuronal degeneration through the degradation of neurolemmal, organelle, and cytosolic macromolecules and promoting the mitochondrial release of cytochrome c, which in turn activates and liberates caspase mediated apoptotic cascades (Yagami et al., 2019). Ischemia mediated amplification of TRPM7 currents may thus effectively generate neuronal injury through the described mechanisms.

Mechanosensitive properties of TRPM7 channels (Numata et al., 2007; Song et al., 2014) indicate a putatively important role in mediating the vascular smooth muscle cell cationic waves contributing to generating the regular and rhythmic oscillatory contraction and relaxation constituting the cerebral vasomotion. The oscillations of the cerebral microvasculature propel bulk flow of fluid within the perivascular compartment, a microcirculatory mechanism serving to nourish neurons and remove waste products (Van Veluw et al., 2020). Hypoxia mediated neuronal depolarization (Revah et al., 2019) depletes the extracellular compartment and synaptic cleft of divalent cations (Lin et al., 2004), generating a compensatory enhancement of intracellularly directed cationic flux through TRPM7 channels (Sun et al., 2009). The contribution of the cerebral vasomotion to regulating the cerebral microcirculation and perivascular fluid compartment bulk flow remains to be further elucidated. Accordingly, several compounds effectively mitigating nerve injury in various settings may exhibit correspondingly major or principal effects upon the activity TRPM7 channels.

Treatment with nafamostat mesylate may alternatively enhance or antagonize ionic flux through TRPM7 channels via competition with divalent cations in the presence of low extracellular concentrations of calcium and magnesium. Nafamostat mesylate reduces neuronal excitability and effectively prevents TRPM7-mediated rises in intracellular calcium in cultured hippocampal and cortical neurons, though amplifies these currents in the presence of extracellular divalent cations concentrations within the physiological range (Chen et al., 2010b). Though pre-exposure to nafamostat mesylate potentiates TRPM7 currents, treatment with the agent effectively blunts the paradoxically low extracellular calcium-mediated enhancement of intracellularly directed calcium flux. Nafamostat mesylate coordinately attenuates the activity of acid sensing ion channels (Chen et al., 2010c). A variety of neuropathologic processes generate cellular hypoxia and tissue acidosis, potently modulating the activity of acid-sensing ion channels (Zhang et al., 2020a). Authors have consequently implicated these channels in mediating neuronal injury in models of autoimmune encephalomyelitis (Friese et al., 2007; Fazia et al., 2019; Mazzocchi et al., 2019), cerebral ischemia (Savic Azoulay et al., 2020), and neuropsychiatric dysfunction (Faraci et al., 2019). These mechanisms provide an evidence base supporting the potential clinical utility of exploiting these agents to treat a variety of neurological dis-

orders (Barua et al., 2019).

Nafamostat Mesylate Modulates BDNF/TrkB/ERK1/2/CREB Signaling

The BDNF/TrkB/ERK1/2/CREB pathway plays a critical role in maintaining neuronal survival (Liu et al., 2017). BDNF activates the receptor tyrosine kinase, TrkB, which phosphorylatively activates the pleiotropically active extracellular regulated kinase 1/2 (ERK1/2) (**Figure 7**). Activation of BDNF/TrkB/ERK1/2 and adenylate cyclase/cyclic AMP/protein kinase A pathways commensurately activate the nuclear transcriptional activity of CREB. Cyclin dependent kinase 5 phosphorylates and activates TrkB and MEK1 (Kansy et al., 2004; Cheung et al., 2007; Lai et al., 2012; Modi et al., 2012; Mishiba et al., 2014; Gutierrez-Vargas et al., 2015). Compromise of the described signaling cascades may thus putatively reduce neuronal resilience to ischemic and other forms of neurocellular injury. Nafamostat mesylate coordinately upregulates signaling via TrkB and extracellular regulated kinase (ERK) 1/2 and the expression of cyclic adenosine monophosphate regulatory element binding (CREB) protein in experimental middle cerebral artery territorial ischemia (Liu et al., 2017). The serine protease inhibitor nafamostat mesylate contemporaneously downregulates expression of thrombin and prevents thrombin mediated activation of cyclin-dependent kinase 5 (Cdk5) (Liu et al., 2017). Nafamostat mesylate mediated amplification of these pathways thus contributes to promoting neuronal survival. In contrast, p38 and Janus kinase (JNK) exacerbate ischemia-induced apoptosis (Lai et al., 2014).

Nafamostat Mesylate Modulates NF- κ B Signaling

Treatment with nafamostat mesylate coordinately inhibits NF- κ B signaling and amplifies glycogen synthase kinase 3 β signaling (Haruki et al., 2017). These effects may be generated both directly and indirectly through the activation of protein phosphatase 2A inhibitor, which effectively prevents protein phosphatase 2A from dephosphorylating and inactivating glycogen synthase kinase 3 β (Haruki et al., 2017). Glycogen synthase kinase 3 β inhibitor contemporaneously inhibits NF- κ B and GSK-3 β signaling (Haruki et al., 2017). The binding of TNF- α to its cell surface membrane receptor preferentially modulates the assembly of a receptor scaffold, variably comprised of RIP1, TRADD, TRAF2, FADD, and cIAP1/2. RIP1 only undergoes ubiquitylation in the presence of cIAP1/2. Consequently, in the absence of cIAP1/2, the tumor necrosis factor receptor scaffold preferentially activates the proapoptotic enzyme caspase 8, which in turn activates caspase 3. Activated caspase 3 consequently activates polyadenosine ribose polymerase, leading to cellular apoptosis (Haruki et al., 2017). In the presence of cIAP2, RIP1 undergoes ubiquitylation leading to the activation of I κ Ks mediating phosphorylative activation of I κ B α (Haruki et al., 2017). These effects collectively prevent NF- κ B from generating signaling via the canonical pathway and non-canonical pathways (Haruki et al., 2017). Under normal con-

ditions, activation of the canonical and non-canonical pathways through NF- κ B generates transcriptional upregulation of proteins enhancing cellular proliferation and synthesis of VEGF, ICAM-1, and matrix metalloproteinase-9 (Haruki et al., 2017), the upregulation of which coordinately promotes tumor cell invasion and adhesion. Nafamostat mesylate also inhibits tryptase from activating PAR-2, a potent activator of cellular proliferation, and inhibits ANGPT1, which activates TIE2, a mediator of cellular proliferation and angiogenesis. These effects contribute to reducing tumor cell proliferation rate and inhibiting apoptosis.

Nafamostat Mesylate Reduces the Endoplasmic Reticulum Stress Response

The endoplasmic reticulum stress response plays a critical role in the pathogenesis (i.e., development and initiation of a pathologic perturbation of normal physiology) and pathophysiology (i.e., emergent evolution of pathologic sequelae of the initial perturbation of normal physiology) of ischemia mediated cellular and neuronal degeneration (Doycheva et al., 2019). The ischemia induced endoplasmic reticulum stress response generates reactive oxygen species (Ke et al., 2020), increases cytosolic calcium concentration (Mohsin et al., 2020), and activates proapoptotic signaling cascades (Bánhegyi et al., 2007; Fernández et al., 2015). Cellular ischemia briskly enhances the expression of the endoplasmic reticulum stress response proteins glucose-regulated protein 78 (GRP78) (Avila et al., 2013), CATT/EBP homologous protein (CHOP) (Taniguchi and Yoshida, 2015), and phosphorylated-eukaryotic initiation factor 2 α (eIF2 α) (Morimoto et al., 2007; Duan et al., 2010; Nakka et al., 2010). In rats experimentally subjected to transient middle cerebral artery territorial ischemia, nafamostat mesylate powerfully reduces the expression of endoplasmic reticulum stress response proteins glucose-regulated protein (GRP78), CHOP, and eIF2 α (Kwon et al., 2015), paralleling gross reductions in cerebral infarct volume and edema, neuronal degeneration, and microglial and astrocytic activation. CHOP, a critical mediator of the endoplasmic reticulum stress response, may be subjected to regulation by PERK-eIF2 α -ATF4 pathway via an unfolded protein response (Zhang and Kaufman, 2008). Nafamostat mesylate attenuates the ischemia induced endoplasmic reticulum stress response and subsequent activation of eIF2 α effectively preventing consequent activation of ATF4 and CHOP (Zhang and Kaufman, 2008). Accordingly, by extrapolative deduction and empirical validation, the endoplasmic reticulum stress response mediated amplification of proapoptotic pathways may represent a useful biomolecular strategy protecting the cerebrum (Tajiri et al., 2004). Nafamostat mesylate attenuates the endoplasmic reticulum stress response, an effect contributing to the neuroprotective effects mediated by this compound (Kwon et al., 2015).

Nafamostat Mesylate Modulates Pathways Implicated in Carcinogenesis

Authors have extensively investigated the utility and molec-

ular effects of nafamostat mesylate in models of melanoma (Komi and Redegeld, 2019) and breast (Mander et al., 2018; Komi and Redegeld, 2019), pancreatic (Furukawa et al., 2010; Haruki et al., 2017; Chen et al., 2019a) and colorectal (Brandi et al., 2012; Komi and Redegeld, 2019) adenocarcinoma. Treatment with nafamostat mesylate sensitizes tumor cells to the cytotoxic effects of chemotherapy (Uwagawa et al., 2009a, b; Fujiwara et al., 2011a, 2013; Gocho et al., 2013; Haruki et al., 2013a, b; Horiuchi et al., 2016; Lu et al., 2016; Shirai et al., 2016; Sugano et al., 2018) and prevents radiotherapy induced upregulation of NF- κ B. Nafamostat mesylate effectively reduces KRAS, BRAF, and PIK3CA oncogene mediated signaling (Brandi et al., 2012). Nafamostat mesylate downregulates NF- κ B mediated signaling, dampens inflammatory cytokine release (Li et al., 2016), blunts interferon γ mediated upregulation of programmed death ligand 1 (Homma et al., 2018), effectively preventing tumor cell immune evasion (Waki and Yamada, 2016), and potentiates cytotoxic CD8 T cell adaptive cellular immunity (Li et al., 2009). The serine protease activity of nafamostat mesylate extends its utility to treat patients suffering from multiple myeloma. Accordingly, use of this agent exhibits similar therapeutic efficacy compared with the proteasome inhibitor bortezomib in treating this clonal plasma cell dyscrasia. Compared with bortezomib, which preferentially distributes to blood and bone marrow compartments, nafamostat mesylate exhibits a pharmacokinetic advantage given specific distribution to the solid tumor mass (Manasanch and Orłowski, 2017). The recent discovery of nafamostat mesylate mediating inhibitory modulation of the demethylase activity of the fat mass obesity associated protein indicates the existence of an extraordinarily broad range of pleiotropic effects underlying the therapeutic potency of this agent in the treatment of carcinomas and a spectrum of diseases and conditions (Chen and Du, 2019; Han et al., 2019b).

Broadening Clinical Applications of Nafamostat Mesylate

Preclinical (Sawa et al., 1998; Altshuler et al., 2012; Gobetti et al., 2012; Chen et al., 2014, 2019b; Kwon et al., 2015; Liu et al., 2017) and clinical (Hirota et al., 2020; Minakata et al., 2019) studies continue to diversify and broaden the range of pathological conditions for which nafamostat mesylate may be effectively and safely used. These investigational indications include treatment and/or mitigation of lipopolysaccharide-induced endothelial injury in the lungs (Sawa et al., 1998; Hagiwara et al., 2007), systemic inflammatory response syndrome, sepsis, severe sepsis, septic shock, hemorrhagic shock (Altshuler et al., 2012), chorioamnionitis (Nakatsuka et al., 2000), ischemia/reperfusion injury in thromboembolic or transplant-related organ ischemia (e.g., heart, liver, pancreas, kidney), allograft rejection, graft versus host disease, and cancer (Chen et al., 2019a). Treatment with nafamostat mesylate mitigates the neurodegenerative sequelae of thromboembolic, occlusive, or transplant related myocardial, hepatic, renal, and intestinal ischemia (Schwartz et al., 2008; Gobetti et al., 2012) and autoimmune encephalomyelitis

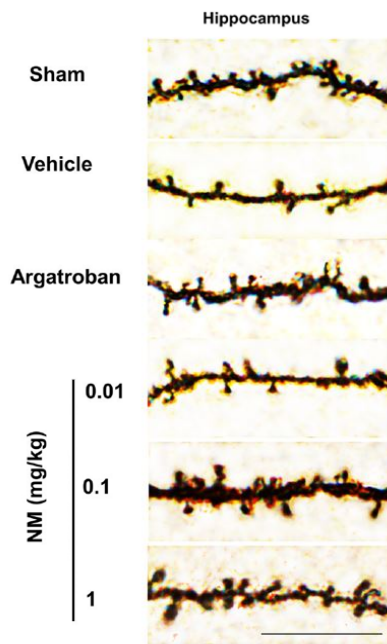


Figure 4 Nafamostat mesylate (NM) attenuates middle cerebral artery occlusion induced synaptic neuroarchitectural reorganization in the hippocampus.

Middle cerebral artery territorial ischemia causes dendritic pruning and downregulation of postsynaptic density protein of molecular weight 95 kilodaltons and synaptophysin. These effects are attenuated by NM. Scale bar: 5 μ m. Modified with permission from Figure 5 of Liu et al. (2017).

(Li et al., 2009). In preclinical animal models, treatment with nafamostat mesylate effectively reduced lipopolysaccharide-induced injury to lung endothelium (Hagiwara et al., 2007) and enhanced survival following liver transplantation (Miyagi et al., 2009; Ryu et al., 2011). In mice infected with *Escherichia coli* O157:H7 and treated with TNF- α to amplify host inflammatory responses, nafamostat mesylate effectively reduced mortality and neurological pathology, prevented glomerular injury, and reduced the expression of proinflammatory cytokines (Isogai et al., 1998; Duan et al., 2018). Treatment with nafamostat mesylate was shown to effectively improve motor recovery in Yucatan minipigs sustaining hemorrhagic shock and mesenteric ischemia (Kim et al., 2010). Nafamostat mesylate prevented the cellular ischemia resulting from reperfusion injury, which ensues following surgical revascularization of, and restitution of blood flow to, pancreatic allogeneic transplants (Shimizu et al., 1995; Urushihara et al., 1996). Nafamostat mesylate may have therapeutic benefit in treating solid organ malignancies, with cytotoxic effects chiefly generated through the induction of cancer cell apoptosis mediated by activation of caspase 8 (Furukawa et al., 2010; Chen et al., 2019a).

Conclusions

Treatment with nafamostat mesylate significantly and consistently attenuates gross and microarchitectural nerve injury and improves functional recovery in preclinical animal models of ischemic cerebral infarction, intracranial hemorrhage, and subarachnoid hemorrhage (Yanamoto et al., 1992, 1994a,

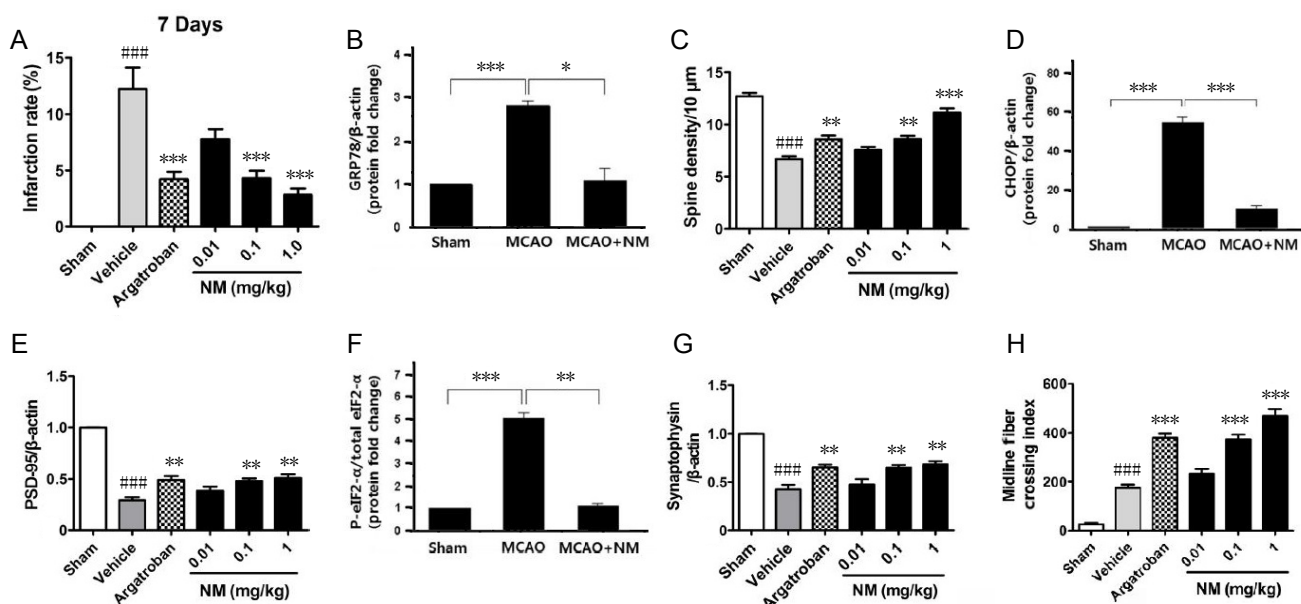


Figure 5 Effects of nafamostat mesylate (NM) on middle cerebral artery territorial ischemia across several studies.

(A) NM reduced cerebral infarct volume in a middle cerebral artery occlusion model of cerebral ischemia (Liu et al., 2017). (B) NM reduced dendritic pruning (Liu et al., 2017). (C, D) NM reduced ischemia mediated decreases of postsynaptic density protein 95 (PSD-95) (C) and synaptophysin (D); Liu et al., 2017). (E, F) NM reduced ischemia mediated augmentation of the glucose-regulated protein 78 (GRP78)/beta actin (E) and CATT/EBP homologous protein (CHOP)/beta-actin ratios (F; Kwon et al., 2015). (G) NM reduced ischemia mediated augmentation of the phosphorylated eukaryotic initiation factor 2 alpha (p-eIF2 α)/total eukaryotic initiation factor 2 alpha (eIF2 α) ratio (Kwon et al., 2015). (H) NM augmented ischemia mediated increases of fibers decussating across the midline (Liu et al., 2017). Modified with permission from Liu et al. (2017), Kwon et al. (2015), and Chen et al. (2014).

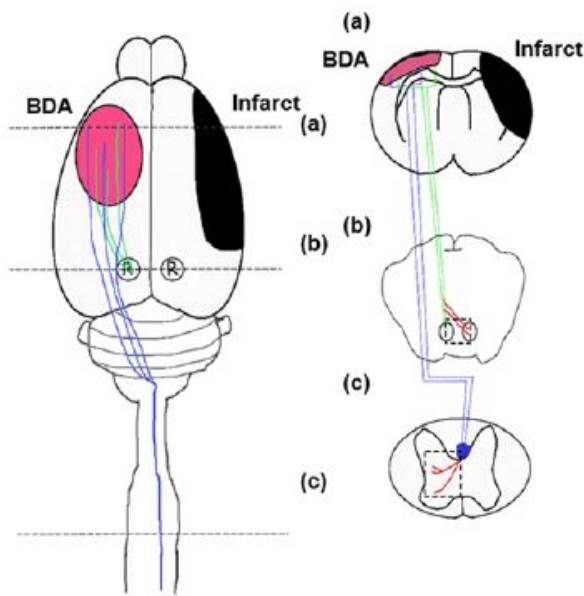


Figure 6 Nafamostat mesylate promotes regeneration of corticobulbar and corticospinal decussating fibers.

Middle cerebral artery territorial ischemia causes infarction of sensorimotor cortex and compromises pyramidal tract projections to brainstem cranial motor nuclei and spinal motoneurons and interneurons. Wallerian degeneration ensues following infarction of the neuronal somata comprising the pyramidal tract in primary motor and primary somatosensory cortices. The spared contralateral pyramidal tract provides decussating fibers reinnervating denervated brainstem and spinal motor nuclei (red fibers in schematic). The regrowth of decussating fibers occurs at the level of the reinnervated motoneurons. The intact pyramidal tract of the contralateral hemisphere was traced utilizing the anterograde label biotinylated dextran amine. Treatment with argatroban and nafamostat mesylate augmented the total crossing fibers, length of crossing fibers, and gap junction associated protein-43 (GAP-43). (a) Coronal section through the forebrain inclusive of cerebral infarct region and uninjured primary motor cortex. Cortical pre-motor interneurons project from primary motor cortex towards midbrain (green pathways) and spinal cord (blue pathways) levels. (b) Transverse section through the midbrain at the level of the red nuclei. Monosynaptic corticobulbar axon-dendritic and axosomatic inputs conveyed to neurons of the ipsilateral red nucleus sprout axons innervating the contralateral red nucleus (red pathways). (c) Transverse section through the cervical spinal cord. Axons coursing through the contralateral corticospinal tract sprout collateral fibers decussating at the level of the spinal cord and innervating the contralateral ventral horn (red pathways). BDA: Biotinylated dextran amine; R: red nucleus. Modified with permission from Figure 4 of Liu et al. (2017).

b; Zhang et al., 2001; Chen et al., 2014; Kwon et al., 2015; Liu et al., 2017; Ghali et al., 2018). Mechanisms underlying these effects include inhibition of serine protease enzymatic catalytic activity (Chen et al., 2014), modulation of intracellular signal transduction pathways (Collo et al., 2014; Haruki et al., 2017), attenuation of neuroinflammatory responses (Isogai et al., 1998), reduction in ischemia-induced blood-brain barrier disruption (Machida et al., 2017), reduced expression of endoplasmic reticulum stress proteins (Kwon et al., 2015), and modulation of ionic fluxes through TRPM7 (Chen et al., 2010a) and acid sensing ion (Chen et al., 2010b) channels. Nafamostat mesylate amplifies the endothelial release of nitric oxide through potentiation of Akt signaling (Choi et al., 2016), an effect which may prove to have potential utility in treating various cardiovascular diseases. Modulation of nitric oxidergic elaboration by nafamostat mesylate may potentially modulate the cerebral vasomotion, a mechanism which could figure prominently in the pathogenesis of microcirculatory

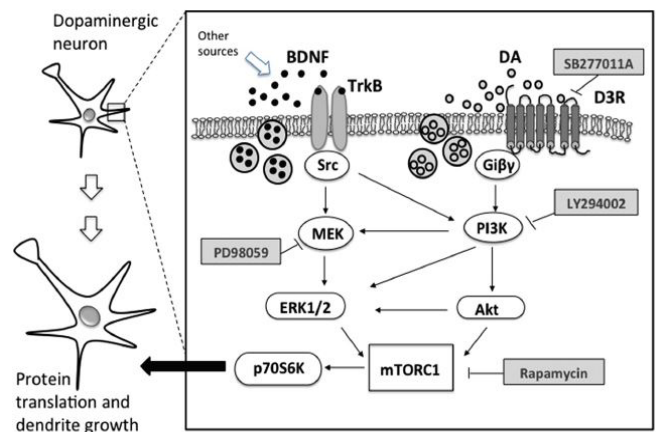


Figure 7 BDNF-TrkB signaling.

The schematic illustrates BDNF signaling. BDNF mediates its cellular effects by binding to the cell membrane homodimeric TrkB receptor. TrkB activation stimulates Src activity, which activates MEK and PI3K. MEK activates extracellular regulated kinase 1/2 (ERK1/2), which activates the mTORC1 and CREB (not shown). PI3K is activated by G protein coupled receptor signaling and activates its protein kinase substrates MEK, ERK1/2, and Akt. In this diagram, dopamine binding to the D3 dopamine receptor potentiates PI3K activity through G protein mediated signaling. The kinase activities of MEK and ERK1/2 are both positively modulated by the kinase activity of PI3K. Akt stimulates ERK1/2 and mTORC1 activity. Activation of mTORC1 converges on potentiating p70S6K promoted protein translation and growth of neural elements. Various pharmacological agonists prove useful in molecularly dissecting these pathways. SB277011A inhibits the D3 dopamine receptor, LY294002 inhibits PI3K activity, PD98059 inhibits MEK activity, and rapamycin inhibits mTORC1. Modified with permission from Collo et al. (2014). Akt: Protein kinase B; BDNF: brain-derived neurotrophic factor; CREB: cAMP response element binding protein; DA: dopamine; D3R: dopamine 3 receptor; ERK: extracellular regulated kinase 1/2; Giβγ: subunit of Gi protein; MEK1/2: mitogen-activated protein kinase/extracellular regulated kinase 1/2; mTORC1: mammalian target of rapamycin 1; PI3K: phosphatidylinositol 3-kinase; Src: non-receptor tyrosine kinase of the Src family of tyrosine kinases; TrkB: neurotrophic tyrosine kinase receptor type 2.

dysfunction in subarachnoid hemorrhage related vasospasm, with amelioration of the same generating therapeutic benefit (Westermaier et al., 2009). The plethora of data gathered in preclinical animal models (Chen et al., 2014; Kwon et al., 2015; Liu et al., 2017) thus collectively demonstrates a powerful set of neuroprotective effects mediated by nafamostat mesylate, extending its utility to the clinical arena in the management of ischemic cerebral infarction, intracranial hemorrhage, and subarachnoid hemorrhage (Chen et al., 2014, 2019a; Kwon et al., 2015; Liu et al., 2017). The preclinical evidence base generated by these studies strongly supports and validates future studies designed to evaluate the clinical utility of these agents in the treatment of neurovascular diseases in human clinical trials (Chen et al., 2019a, b, 2020). Burgeoning evidence continues to accrue supporting a prominent role of these agents across a wide host of clinical applications, including the mitigation of thromboembolic, occlusive, and transplant related myocardial, intestinal, hepatic, and renal ischemia, septic shock, alloimmune hyperacute, acute, and chronic graft rejection, and acute graft versus host disease (Chen et al., 2019a). Further studies are necessary to more thoroughly elucidate and dissect the precise mechanistic effects mediated by nafamostat mesylate, gabexate mesylate, and argatroban upon intracellular signal transduction and the efficacy and safety of utilizing these compounds to treat various human diseases.

Author contributions: Conception and design, acquisition of data, analysis and interpretation of data, drafting article and revising critically for intellectual content; and approval of final manuscript: GZG, MGZG.

Conflicts of interest: We have no conflicts of interest to disclose.

Financial support: None.

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Data sharing statement: Datasets analyzed during the current study are available from the corresponding author on reasonable request.

Plagiarism check: Checked twice by iThenticate.

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