Tohoku J. Exp. Med., 2015, **237**, 45-50

Medical Hypothesis

A Synthetic Serine Protease Inhibitor, Nafamostat Mesilate, Is a Drug Potentially Applicable to the Treatment of Ebola Virus Disease

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Ebola virus disease (EVD) has been a great concern worldwide because of its high mortality. EVD usually manifests with fever, diarrhea and vomiting, as well as disseminated intravascular coagulation (DIC). To date, there is neither a licensed Ebola vaccine nor a promising therapeutic agent, although clinical trials are ongoing. For replication inside the cell, Ebola virus (EBOV) must undergo the proteolytic processing of its surface glycoprotein in the endosome by proteases including cathepsin B (CatB), followed by the fusion of the viral membrane and host endosome. Thus, the proteases have been considered as potential targets for drugs against EVD. However, no protease inhibitor has been presented as effective clinical drug against it. A synthetic serine protease inhibitor, nafamostat mesilate (NM), reduced the release of CatB from the rat pancreas. Furthermore, it has anticoagulant activities, such as inhibition of the factor VIIa complex, and has been used for treating DIC in Japan. Thus, NM could be considered as a drug candidate for the treatment of DIC induced by EBOV infection, as well as for the possible CatB-related antiviral action. Moreover, the drug has a history of large-scale production and clinical use, and the issues of safety and logistics might have been cleared. We advocate *in vitro* and *in vivo* experiments using active EBOV to examine the activities of NM against the infection and the DIC induced by the infection. In addition, we suggest trials for comparison among anti-DIC drugs including the NM in EVD patients, in parallel with the experiments.

Keywords: cathepsin B; disseminated intravascular coagulation; Ebola virus disease; nafamostat mesilate; surface glycoprotein
Tohoku J. Exp. Med., 2015 September, **237** (1), 45-50.

Introduction

Ebola virus disease (EVD) has been causing repeated outbreaks and epidemics since 1976 in Central African countries, such as the Congo, Sudan, and Uganda, and has accounted for many casualties. The recent EVD outbreak emerged in Guinea in December 2013 and caused epidemics in 2014 in several Western African countries, primarily Guinea, Sierra Leone and Liberia. EVD is still a great threat to the local people and healthcare staff, and it is of great concern worldwide. EVD manifests with several symptoms such as fever, diarrhea and vomiting (Feldmann et al. 2013), as well as systemic hemorrhage and disseminated intravascular coagulation (DIC) (World Health Organization 2014; Centers for Disease Control and Prevention 2015). To date, there is neither a licensed Ebola vaccine nor an effective therapeutics, and the treatment is currently limited to supportive management. Numerous vaccines and agents including convalescent plasma or recombinant antibodies, small inhibitory RNA and antivirals, have become candidates for the prevention and treatment because of their effectiveness in animal experiments (World Health Organization 2014). Some of them have already been compassionately tried for patients, and further evaluations through official clinical trials are planned (Mohammadi 2014; Marzi and Falzarano 2015). However, the supply of drugs with stable quality and quantity, i.e. the logistical affairs associated with treating many patients, are of another concern and may become obstacles for such agents to be widely used, even if any of them prove to be effective.

Ebola virus (EBOV), which belongs to family *Filoviridae*, is an enveloped virus that has a non-segmented

Received May 8, 2015; revised and accepted August 11, 2015. Published online September 3, 2015; doi: 10.1620/tjem.237.45.
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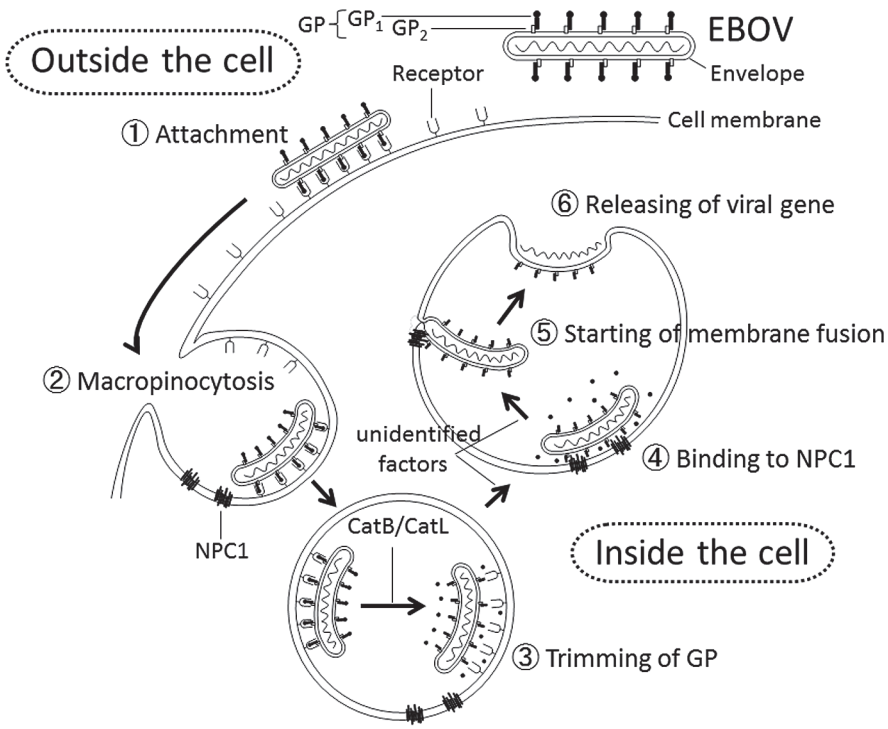

Fig. 1. A schematic diagram of the entry process of EBOV into the host cells. Binding to the receptor on the host cell surface (1). Internalization by macropinocytosis into the endosome (2). The endosomal cysteine proteases, CatB/CatL trim $GP_{1,2}$ heterodimer in the acidic environment that is present inside the late endosome/lysosome (3). The trimmed GP_1 binds to the endosomal/lysosomal membrane protein, NPC1 (4). The fusion is triggered by unknown lysosomal factor(s) (5). The viral gene is released into the cytoplasm of the cell in the form of nucleocapside (6). NPC1: Niemann-Pick C1.

negative-sense RNA genome. EBOV has a heterodimeric glycoprotein (GP) (= EBOV GP) on its surface that consists of GP_1 and GP_2 held together by a disulfide bond (Feldmann et al. 2013) (Fig. 1). In other words, EBOV GP consists of GP_1 and GP_2 that are cleaved from the nascent GP (GP_0) by the action of a subtilisin-like proprotein convertase, furin, that occurs in the Golgi apparatus.

Virus replication starts after the attachment of the virus on the cell surface with EBOV GP followed by internalization into the endosome, where the fusion of the viral membrane with the endosomal membrane leads to the release of its genome into the cytoplasm (Fig. 1) (Hofmann-Winkler et al. 2012; Hunt et al. 2012; Feldmann et al. 2013). The GP_1 and GP_2 are processed by several endosomal proteases, including cysteine proteases cathepsin B (CatB) and cathepsin L (CatL), which are active in the acidic environment that is made inside the late endosome/lysosome. The processing is considered to prime the fusion as shown in Fig. 1 (Chandran et al. 2005). In brief, a substantial portion of GP_1 is initially removed by CatL, which is then trimmed into a smaller form by CatL and/or CatB and is cleaved to a still smaller form by CatB. The trimmed GP_1 binds to the endosomal/lysosomal membrane protein, Niemann-Pick C1 (NPC1), which is essential for fusion (Hofmann-Winkler et al. 2012; Hunt et al. 2012; Feldmann et al. 2013). The binding also requires third lysosomal factor that has yet to be identified, and after the binding occurs, an additional unknown stimulus triggers the fusion activity of GP_2.

This processing, particularly that by CatB and CatL, has suggested that interfering with cathepsin activity could represent a possible therapeutic strategy against EBOV infection (Chandran et al. 2005; Hunt et al. 2012). Nafamostat mesilate (NM), (6-amidinonaphthalen-2-yl 4-guanidinobenzoate bis (methanesulfonate)) is a synthetic serine protease inhibitor (Fujii and Hitomi 1981). It inhibits a broad range of proteases (Aoyama et al. 1984) and was shown to suppress the leakage of CatB from lysosomes in rat models of acute pancreatitis (Manabe et al. 1992). NM has been used clinically for the treatment of pancreatitis in Japan (Toya et al. 1984) and was approved by the U.S. Food and Drug Administration as the generic name, Nafamostat Mesylate. Considering the effect of NM on CatB (Manabe et al. 1992), we propose that NM might present a candidate drug to inhibit EBOV infection. Indeed, CatB is thought to be a major protease required for the proteolytic processing of EBOV GP (Chandran et al. 2005).

In addition, NM improved DIC in the experimental animal models (Yoshikawa et al. 1983; Koshiyama et al. 1987), and had anticoagulant effects *in vitro* (Uchiba et al. 1994) and in humans (Koshiyama et al. 1984; Hitomi et al. 1985). In this connection, NM has been used for the treatment of DIC (Shibata et al. 1988; Okamoto et al. 1991), as

well as for extracorporeal circulation (Tsukagoshi et al. 2000) and hemodialysis (Pak et al. 1988) as an anticoagulant. Thus, NM has been used widely in Japan (Japanese Society of Hospital Pharmacists 2015). DIC is a serious complication of EVD (World Health Organization 2014; Centers for Disease Control and Prevention 2015), and NM could represent a possible drug against DIC induced by EBOV infection, as well.

Such off-label use of the drug has advantages over developing a new drug in terms of saving the time until application to humans and of the logistics: the clinical use of an already approved drug for symptomatic treatment would not be associated with any major ethical problems, although there may have some restrictions.

Thus, we advocate a two-pronged strategy: *in vivo* experiments on both the antiviral and anti-DIC activities of NM using active EBOV in the highest biosafety level laboratories and a comparison of the efficacy of the drug for improving DIC in EVD with other anti-DIC drugs in affected patients.

Hypothesis

NM has two possible mechanisms of action as a drug candidate (Fig. 2).

1. Effects on CatB and EBOV replication

The multiple processing of the EBOV GP inside the late endosome/lysosome by proteases including CatB and CatL, which appear to be critical for priming the membrane fusion, may represent a therapeutic target for EBOV replication. Chandran et al. (2005) performed *in vitro* experiments in the Vero cell line derived from the African green monkey kidney using recombinant vesicular stomatitis viruses expressing EBOV GP and showed that cysteine proteases are important for the proteolytic processing of the EBOV GP in the endosome, which facilitate EBOV entry into the cells, and CatB plays an essential role in this process. It was demonstrated that a synthetic CatB inhibitor, CA074, suppressed the proteolysis and viral replication following infection with the Zaire strain of EBOV (ZEBOV) (Chandran et al. 2005). These findings presented that protease inhibitors that can effectively suppress CatB may be beneficial for treatment of EVD.

In contrast, Marzi et al. (2012) recently demonstrated that CA074 inhibited the entry of ZEBOV into Vero E6 cells but did not inhibit the replication inside cells, and that a mouse embryonic fibroblast cell lines lacking CatB supported the replication of ZEBOV. Furthermore, CatB knockout mice were equally susceptible to a challenge with the lethal dose of the mouse-adapted ZEBOV as the control wild-type mice (Marzi et al. 2012). These findings suggested that the processing of EBOV GP could be mediated by an array of proteases other than CatB, as well, and that CatB may not be exclusively required for EBOV infection. A possible explanation for them might come from the study by Wong et al. (2010), who reported an *in vitro* infection experiment showing that CatB is required but insufficient for the entry of ZEBOV, which was confirmed using a *forward genetic* system using a recombinant VSV (rVSV) encoding a mucin domain-deleted ZEBOV GP in the place of VSV glycoprotein G. The recombinant virus could replicate in the cell, even when the CatB activity was blocked with the protease inhibitor CA074. They found that mutations occurred in GP and suggested that the mutated GP became susceptible to proteolysis by unknown protease(s) other than CatB and CatL, which facilitated subsequent membrane fusion. In other words, EBOV may be able to

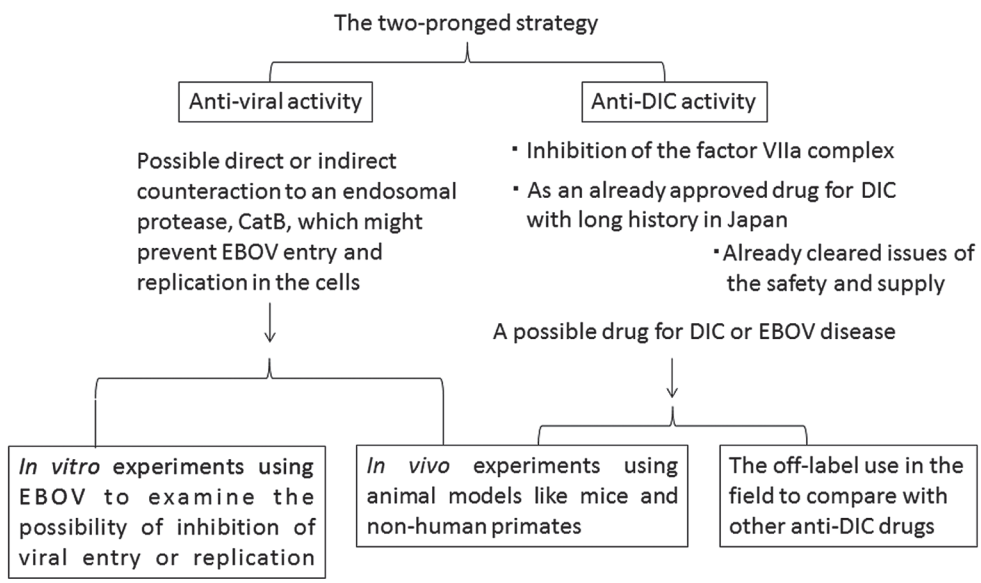

Fig. 2. Schematic summary of possibilities of nafamostat mesilate as a candidate drug for EBOV disease and the strategy for examining the possibilities. The two-pronged strategy could be suggested.

use some CatB-independent pathway under certain conditions. Such a bypass phenomenon might also have occurred in the CatB knockout mice (Marzi et al. 2012). However, the findings reported by Wong et al. (2010) have not been experimentally confirmed with live EBOV. Likewise, there has been no further study to determine whether a mutant virus which might use the CatB-independent pathway was actually generated *in vivo* (Marzi et al. 2012).

The contradictory findings of the studies by Chandran et al. and Marzi et al. should be carefully interpreted. Chandran et al. (2005) suggested that NM could suppress the EBOV replication *in vivo*; in contrast, Marzi et al. (2012) suggested that NM could not suppress the viral replication in a non-human primate or humans. Therefore, whether NM can suppress the EBOV viral replication should be examined with a series of *in vitro* and *in vivo* experiments in a variety of models. In addition, the study by Wong et al. (2010) suggests that caution should be taken regarding the emergence of CatB-independent mutant EBOV during the course of treatment with this drug, even if it is found to be effective.

2. Management of DIC in EVD

Experimentally, NM reduces the mortality of the endotoxin-induced DIC in rats (Yoshikawa et al. 1983; Koshiyama et al. 1987), and also improves DIC caused by circulatory arrest in dogs (Takemoto et al. 1986). And NM inhibits kallikrein (Aoyama et al. 1984) and the factor VIIa complex (Uchiba et al. 1994), one of proteases associated with the pathogenesis of DIC, and has other anti-coagulant effects (Hitomi et al. 1985). This drug has been widely used in the clinical setting in Japan for the treatment of DIC (Okamoto et al. 1991). Geisbert et al. (2003) reported that the survival rate of a non-human primate in the EVD animal model increased by treatment with a recombinant inhibitor of VIIa/tissue factor. Thus, NM may also improve the survival rate of patients with EVD.

Testing the hypothesis

The following approaches (Fig. 2) may be used to test the aforementioned hypotheses:

1. Experimental approach: *In vitro* experiments are recommended using tissue culture and pseudotype or recombinant virus carrying EBOV GP (Chandran et al. 2005; Hunt et al. 2012; Feldmann et al. 2013) or active EBOV, to investigate whether NM inhibits the proteolysis of GP and the viral genome entry into the cell, and the subsequent replication of EBOV, respectively. Apart from the above, the possible inhibition of EBOV replication and tissue damage by NM through its possible indirect action of inhibiting the release of CatB should also be investigated using *in vivo* animal models (Chandran et al. 2005; Marzi et al. 2012). In parallel, experiments using animal models are also necessary to investigate the effects of NM on EBOV-induced DIC. Mice, guinea pigs and non-human primates could represent relevant models because EBOV infection induces thrombocytopenia in all three of these animals and non-human primates develop DIC (Bray et al. 2001). Pharmacologically, plasma concentrations of NM were maintained at significant levels in the lungs and kidneys (0.13 and 0.25 μg/mL, respectively, at 4 h after intravenous injection (1 mg/kg)) in rats (Nanpo et al. 1984).

2. Administration of NM as an off-label drug to EVD patients: To treat EVD patients with DIC or a similar condition, the efficacy of NM should be examined by comparing its effects with those of other existing anti-DIC drugs.

About the drug

NM has been used to treat pancreatitis since 1986 and for DIC and extracorporeal circulation since 1989. The drug showed approximately 56% efficacy in a multicenter phase II clinical trial for the treatment of 108 DIC cases (Shibata et al. 1987). The adverse effects of NM were investigated with 6,732 cases of acute pancreatitis, 3,602 cases of DIC, and 4,053 cases of extracorporeal circulation, with the administration of drug doses of 10-40 mg/day or more, 0.05-0.26 mg/kg/h or more and 20-60 mg/h, respectively, and reported in 117 (1.74%), 241 (6.69%) and 48 cases (1.18%), respectively. The most frequently observed abnormalities among these cases were hyperkalemia which occurred in 0.19% of the patients with acute pancreatitis; hyperkalemia and hyponatremia, 4.35% and 0.47%, respectively, of the patients with DIC; nausea, 1.01% of the extracorporeal circulation cases (Japanese Society of Hospital Pharmacists 2015).

The mechanisms underlying the hyperkalemia and hyponatremia were suggested in a previous study (Muto et al. 1994).

Benefits as a candidate drug

Several disease-specific treatments modalities have been reported for the management of EVD (World Health Organization 2014). In addition, supportive therapy, such as the oral intake or intravenous infusion of electrolytic solutions, has been tried. Those management approaches were occasionally effective for improving or supporting the patients' clinical condition. However, additional treatments are required for complicated cases such as those with DIC. In general, the new drug or vaccine development processes cost huge time and money because of many steps required for scientific and ethical clearance in terms of efficacy and safety, even in an urgent situation such as the recent EVD epidemic. Experiments at different levels are required, from the *in vitro* experiments at cellular level through the *in vivo* tests using animals and finally to the studies in humans, which examine suppression of viral replication and toxicity (Fig. 2).

In contrast, the off-label use of drugs which had been approved for other purposes and have been broadly used in the clinical setting has several advantages over the new drug development because they have already cleared most

of the safety issues. Only those under any new clinical conditions or in use in combination with other new drug(s) should be assessed. In addition, such drugs have also already cleared one of logistical issues in terms of the mass-production: the ability for constant production and the supply of high-quality drugs in large quantities, which is a major concern for a new drug and may become an obstacle for the drug to be used widely, even if it is proven to be effective. Thus, as an off-label drug that has non-specific anti-DIC effects, NM may be a good candidate drug for the treatment of EVD if it is shown to be effective, particularly if it is proven to be superior in efficacy to other available drugs. In addition, if the CatB-related anti-EBOV activity is confirmed in *in vivo* animal experiments, NM would be more beneficial than other existing drugs due to its dual mechanisms of action.

Acknowledgments

We would like to thank Dr. Chandra Nath Roy of Tohoku University for careful reading and the English language review, Dr. Etsuko Hatagishi, of the Virus Research Center for the drawing the figure and Dr. Michiaki Masuda, Professor of Dokkyo Medical University and Dr. Morio Homma, ex-Professor of Yamagata University and the emeritus Professor of Kobe University for giving a great clue on the idea of this study and for useful discussion.

Declaration of Interests

Mutsuo Yamaya is a Professor in the Department of Advanced Preventive Medicine for Infectious Disease, Tohoku University Graduate School of Medicine. This department was funded by eight pharmaceutical companies, which are as follows: Kyorin Pharmaceutical Co., Ltd., Abott Japan Co., Ltd., Taisho Toyama Pharmaceutical Co., Ltd., AstraZeneca Co., Ltd, Otsuka Pharmaceutical Co., Ltd., Teijin Pharma Co., Ltd., Toyama Chemical Co., Ltd., and Nippon Boehringer-Ingelheim Co., Ltd.

Conflict of Interest

The authors declare no conflict of interest.

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