For an Application of Protease Inhibitor for the Treatment of Viral Respiratory Infections - Acceptable Concentrations of a Protease Inhibitor Nafamostat and Ammonium Chloride for Direct Administration to the Respiratory Epithelium of Mice

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Research Article

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Abstract

Many enveloped respiratory viruses, including SARS-CoV-2, require host proteases for the infection, and *in-vitro* studies have demonstrated that protease inhibitors suppress viral infection. However, no application of inhibitors for the treatment of viral respiratory infections have been reported. This is because no method has been established to efficiently deliver inhibitors to the respiratory epithelium. This study explores methods to safely deliver a protease inhibitor nafamostat by assessing whether adverse effects occur when nafamostat is administered directly to the respiratory epithelium.

To observe the effect of direct respiratory administration on organisms, inbred mice were intranasally administered the inhibitor solutions under anesthesia. 200µM nafamostat at 20µL/day for 1 week could be administered without any adverse effects. Since 1µM nafamostat is known to suppress the viral entry to cell *in vitro*, 200µM nafamostat is expected to show enough inhibitory effect also in mice.

Ammonium chloride (NH₄Cl) is also known to block the viral entry via endosome. The present study has demonstrated that 74mM NH₄Cl could be also administered in the same manner. Since the NH₄Cl solution at 50mM is known to efficiently suppress the entry of SARS-CoV-2 via endosome *in vitro*, NH₄Cl may be also available to treat viral infection *in vivo*.

Results of the present study encourage the research to apply nafamostat and also NH₄Cl for the treatment of respiratory viral infection, including COVID-19.

Introduction

Enveloped respiratory viruses invade the cells by fusing the viral envelope with the cell membrane through the action of viral fusigenic protein. Most fusigenic proteins are activated with proteases of host organisms, in the manner called “cleavage activation of viral fusion activity” [1-4]. Thus, most respiratory viruses require the specific host protease to infect host animal cells. The severe acute respiratory syndrome corona virus (SARS-CoV) and the SARS-CoV 2 (so-called COVID-19 virus) are no exception[5]. The spike protein S of SARS-CoV and SARS-CoV 2 first bind to ACE2 (angiotensin-converting enzyme 2) on the cell membrane. Thereafter, the transmembrane serine protease 2 (TMPRSS2) on the cell surface cleaves S protein, and the fusion capability of S is unleashed, leading to cell invasion⁴⁻⁶. TMPRSS2 is also essential for many other human respiratory viruses, including influenza [3,4,7,8]. TMPRSS2 is known to be inhibited by camostat mesylate [5,7,8], and recently, an *in vitro* study revealed that nafamostat mesylate (nafamostat) suppressed the cell invasion of SARS-CoV-2 even at an extremely low concentration of 1 µM [9].

Nafamostat is widely used as a protease inhibitor in clinical setting, and the safety of long-term use of nafamostat has been established; moreover, TMPRSS2, a target of nafamostat, is not an enzyme essential for biogenesis and homeostasis [10]. These findings indicate that nafamostat is a potential drug candidate for the treatment of respiratory viral infections.

SARS-CoV and SARS-CoV-2, however, can infect cells even in the absence of TMPRSS2 through an alternative pathway, although the infection efficiency is approximately 1/100 compared with that in the presence of TMPRSS2¹¹. In the alternative pathway, the viruses are engulfed in the endosome and cause the cleavage activation of S by endosomal proteases and acidification, thereby entering the cytoplasm via the endosomal membrane [4,5,12,13]. Principally, this pathway could be blocked with specific inhibitors against endosomal proteases, such as E-64d [5], but this is not available *in vivo* because endosomal proteases are essential for homeostasis in organisms. Instead of the protease inhibitor, ammonium chloride (NH₄Cl) may be available to block this pathway, by temporarily interfering with the acidification of endosomes. Indeed, 50mM NH₄Cl has been shown to efficiently inhibit the invasion of SARS-CoV-2 via endosome *in vitro* [5]. Although NH₄Cl has long been clinically used for controlling the pH of intravenous solutions, no case has been reported in which NH₄Cl is used to suppress viral infections *in vivo*. In this study, we assessed whether adverse effects occurred when nafamostat and NH₄Cl were administered directly to the respiratory epithelium and defined their acceptable concentrations.

Materials And Methods

Mice and reagents

Weanling C57BL/6JJmsSlc female mice (3-week-old) were purchased from Charles River Laboratories Japan, Inc. In each cage five to six mice were raised. Nafamostat mesylate for injection (Nichi-Iko Pharmaceutical Co. Ltd.) was dissolved in 5% glucose solution (as a working solution, 200μM) and used for the experiments after appropriate dilution with saline. NH₄Cl (guaranteed reagent) was purchased from FUJIFILM Wako Pure Chemical Corporation. Isoflurane inhalation solution (Pfizer Japan Inc.) was used for anesthesia.

Intranasal administration with nafamostat and NH₄Cl solutions

Under isoflurane anesthesia, the weanling (3-week-old) and adult (5-week-old) mice were intranasally administered with 2 μL and 20 μL of the solution, respectively, once daily for 1 week. Administration into the nasal cavity is a method generally applied in experiments on influenza infection; it is known that administration of 2 μL of solution leads to the solution remaining in the upper respiratory tract, and administration of 20 μL of solution leads to the solution reaching the lower respiratory tract [7,14].

Details of observation

The mice were observed for 12 to 14 days after the administration into nasal cavity. Each mouse was identified by a marker, and changes in the body weight, fur, and behavior (such as crouching) were observed every day. Changes in the body weight of inbred mice have been used as a health status barometer and as an excellent index in both sensitivity and reproducibility for a long time. If the body weight of mouse decreases at a rate exceeding 20% at the start of the experiment and is unlikely to recover, the mouse would be euthanized.

After the completion of the experiment, the mice were humanely euthanized under heavy anesthesia with isoflurane inhalation. A cardiac puncture was conducted with some group of mice, and Oriental Yeast Co. Ltd. was requested to perform biochemical tests on the mice from whom a sufficient amount of serum was obtained.

Statistical analysis

The program Statcel4 was used for statistical analysis. All variables were tested for distribution normality, and analyzed using a parametric test (i.e., Repeated measure two-factor ANOVA). Both tests were upper side and significance level was set at $P < .01$.

Study site and ethical consideration

This study was conducted experimental settings in a room of the Institute of Experimental Animals, Shinshu University according to the regulations for animal experiments and the guidelines for raising and storing experimental animals and relieving distress at the Shinshu University. Based on the regulations for animal experiments at the Shinshu University, the study was conducted by obtaining experimental design approval (approval number 020040).

Results

Experiment 1: Effect of administration of nafamostat into the nasal cavity

The 200 μM nafamostat solution was sequentially diluted with saline to prepare 50 μM , 12.5 μM , and 3.1 μM nafamostat solutions for intranasal inoculation. Each weanling mouse was administered 2 μL once per day for 1 week. One group (five mice in a cage) was administered saline as a control, and another one was administered 9.3mM NH_4Cl solution, which was a pilot experiment for administering NH_4Cl in the following experiment. The change of mean body weight of each group was shown in Figure 1. All the mice used in this experiment grew steadily, and the final mean body weight at day 14 was approximately 17 g in all groups. The mice showed favorable fur, and active movements. The mice administered NH_4Cl showed a movement of frequently rubbing their nose immediately after the administration. Any other pain symptom due to the administration was not observed under the conditions of Experiment 1. This result suggests that the administration of 200 μM nafamostat into the nasal cavity is acceptable in the weanling mice.

Experiment 2: Effect of the administration of nafamostat 200 μM plus NH_4Cl into the respiratory tract through the nasal cavity

Based on the results of Experiment 1, the 5-old-week adult mice were administered a mixture of 200 μM nafamostat and various concentration of NH_4Cl , 20 μL per day for 1 week in Experiment 2. The concentrations of NH_4Cl in the mixtures were 74mM, 37mM, 18.5mM, and 9.3mM. As a control group, mice were administered normal saline.

Body weight change of individual mouse was expressed with per centage of the body weight at the biggining of Experiment 2 and illustrated in Figure 2. Some mice administered 200 μ M nafamostat plus 74 mM, 37 mM, and 18.5 mM NH_4Cl (refer to Figure 2-A, B, C) showed transiently decreased weight (by 1 g or less) by the 3rd day of administration. However, the mice had a steady weight gain on the 4th day of administration and showed the same weight gain as the control mice at the end of the observation period. The mice administered 200 μ M nafamostat plus 9.3 mM NH_4Cl and the control mice did not show decreased weight and gradually increased in weight (Figure 2-D, E).

In addition to the above groups, one group (three mice) was administered 2x20 μ L of 200 μ M nafamostat plus 74mM NH_4Cl under anesthesia per day, and another group (three mice) 37 μ L of 200 μ M nafamostat alone under anesthesia without anesthesia per day. In the former group the mice resisted the intranasal administration and spat some of the inhaled doses. In the latter group the solution was observed to be running out of the nasal cavity several times, probably because the inoculum volume was too much. All the mice showed favorable fur and active movements and showed a steady weight gain similarly to the pattern of the control mice (data not shown).

Although all mice showed favorable fur and active movements, the mice administered 200 μ M nafamostat plus 18.5 μ M NH_4Cl (Group C) seemed to be docile with low physical activity. To examine their health status more precisely, sera were separated from blood drawn by cardiac puncture after the observation period, and a testing company was requested to perform biochemical tests. The amount of serum required for testing was obtained from four out of five mice in Groups A, C, and E, respectively (refer to the supplemental Table 1).

As shown in the Table 1, two out of four mice administered 200 μ M nafamostat plus 18.5 μ M NH_4Cl (Group C) showed higher liver function markers than the normal ranges. The mice in Group C were also administered 200 μ M nafamostat at 2 μ L for 1 week at the weanling age (refer to the supplemental Table 1) in Experiment 1. The high dose administration at that time and/or the twice administration of high dose of nafamostat throughout Experiment 1 and 2 were considered to have stressed the liver of the mice. Meanwhile, the laboratory data on the mice of Group A were all within the normal ranges similarly to those in the control mice of Group C.

These outcomes reveal that the protease inhibitor, nafamostat at 200 μ M 20 μ L/day for 1 week could be administered directly to the respiratory epithelium of adult mice without any adverse effects, and that 74 mM NH_4Cl could also be administered in the same manner.

Discussions

The above acceptable concentration of nafamostat (200 μ M), is 200-fold higher than the effective *in vitro* concentration of 1 μ M, which inhibits the viral fusion of SARS-CoV-2 efficiently⁹ and the acceptable concentration of NH_4Cl (74mM) adequately covers the effective *in vitro* concentration of 50 mM [5]. Nafamostat is widely used as a protease inhibitor in clinical settings, and ammonium is a metabolite that

is ubiquitous in organisms and is quite well known to be rapidly detoxified in the liver. Nafamostat and NH_4Cl administrations may transiently modify some of the function of organism, but they are not antivirals directly acting on the viruses. Therefore, the virus resistant to nafamostat or NH_4Cl may hardly emerge logically. From this point of view, both reagents can be good candidates for therapeutic drugs for COVID-19.

The next step should be to conduct an experiment of administering the drugs to influenza virus-infected mice to confirm whether the drugs exert therapeutic effects within the acceptable concentration ranges. C57BL mice are known to be sensitive to human influenza viruses [14]. If therapeutic effects are confirmed in C57BL mice, this strategy is highly likely to be developed to combat many respiratory viral infections showing the same infection mode, including COVID-19.

As C57BL mice are sensitive to SARS-CoV infection [14], C57BL mice are likely to be used for experiments of healing of infection with SARS-CoV-2, which utilizes the same receptor ACE2 and the same activating enzyme TMPRSS2.

For the application of nafamostat and NH_4Cl in human, it could be considered to administer as a nasal spray into the nasal cavity and via gargling and mouth-rinsing in the oral cavity in the early phase of infection, and as a nebulizer into the respiratory tract in the middle phases of infection. The method of administration is simple, and nafamostat and NH_4Cl are much cheaper to manufacture than vaccines and antivirals. Confirmation of the therapeutic effect of nafamostat plus NH_4Cl would give great hope to developing countries with inadequate medical facilities.

Unfortunately, Shinshu University, to which the author belongs, has no biosafety level (BSL) 2 facilities enabling animal experiments using influenza viruses. Therefore, we hope that further experiments would be conducted to confirm the effect of nafamostat plus NH_4Cl against influenza virus infection in institutions with BSL2 animal experimental setup, using the data of the present study. Further, it is more desirable to directly confirm the therapeutic effect of nafamostat plus NH_4Cl against COVID-19 in institutions where SARS-CoV-2 can be handled.

Conclusion

The outcomes of this study showed that a protease inhibitor, nafamostat (200 μM) at 20 $\mu\text{L}/\text{day}$ for 1 week, could be administered directly to the respiratory epithelium of adult mice without any adverse effects and that NH_4Cl (74 mM) could be administered in the same manner. If it is confirmed that influenza virus-infected mice can be treated with these drugs, it is highly likely that the drugs can be applied for the treatment of SARS-CoV-2 infection, which invades cells by the aid of the same protease via the same route.

Abbreviations

TP,total protein(g/dL); ALB,albumin(g/dL); BUN,blood urea nitrogen(mg/dL);
Cre,creatinine(mg/dL); Na,sodium(mEq/L); K,potassium(mEq/L); Cl,chlorine(mEq/L);
Ca, calcium (mg/dL); IP,inorganic phosphate(mg/dL); Mg, magnesium(mg/dL);
AST, aspartate aminotransferase(IU/L); ALT,L-alanine aminotransferase(IU/L);
ALP,alkaline phosphatase(IU/L); LDH,lactate dehydrogenase(IU/L);
T-Bil,total bilirubin(mg/dL)

Declarations

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Authors' contributions

S. N. contributed to the conception and design of this study, collected data, performed the statistical analysis, and drafted the manuscript.

Ethics approval and consent to participate

This study was conducted experimental settings in a room of the Institute of Experimental Animals, Shinshu University according to the regulations for animal experiments and the guidelines for raising and storing experimental animals and relieving distress at the Shinshu University. Based on the regulations

for animal experiments at the Shinshu University, the study was conducted by obtaining experimental design approval (approval number 020040).

Consent for publication

Not applicable.

Availability of data and materials

Not applicable

Competing interests

The authors declare that they have no competing interests.

Conflicts of interest

None to declare.

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Figures

Figure 1

The average of body weight curve during Experiment 1 Weanling mice (3-week-old) were intranasally administered nafamostat solutions 2 μ L once per day for 1 week and observed until day 12. Nafamostat 200 μ M (n=5), 50 μ M (n=6), 12.5 μ M (n=5), 3.1 μ M (n=5), and normal saline (n=5) as a control group. 9.3mM NH₄Cl solution (n=5) was administered as a pilot experiment for Experiment 2. until the 12th day. There was no significant difference between the six groups by repeated measure two-factor ANOVA (P=.118275).

Figure 2

Percentage of the body weight changes during Experiment 2 Adult mice (5-week-old) were intranasally administered 20µL mixture of 200µM nafamostat and various concentration of NH₄Cl once per day for a week and observed until day 14. The value of individual mice is plotted in colored lines in graphs (A)-(E), with each individual mouse being indicated by a letter-number code. There was no significant difference among the five groups by repeated measure two-factor ANOVA ($P=0.012627$).

Supplementary Files

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