Nafamostat-interferon-alpha combination suppresses SARS-CoV-2 infection in vitro and in vivo

Aleksandr Ianevski [1]*, Rouan Yao [1], Hilde Lysvand[1], Gunnveig Grødeland [2,3,4], Nicolas Legrand [5], Tanel Tenson [6], Magnar Bjørås [1], Denis E. Kainov [1,6,7]*

[1] Department of Clinical and Molecular Medicine (IKOM), Norwegian University of Science and Technology, 7028, Trondheim, Norway

[2] Research Institute of Internal Medicine, Oslo University Hospital Rikshospitalet, 0372, Oslo, Norway

[3] Institute of Clinical Medicine (KlinMed), University of Oslo, 0318, Oslo, Norway

[4] Section of Clinical Immunology and Infectious Diseases, Oslo University Hospital Rikshospitalet, 0372, Oslo, Norway

[5] Oncodesign, 25 Avenue du Québec, 91140 Villebon Sur Yvette, France

[6] Institute of Technology, University of Tartu, 50411 Tartu, Estonia

[7] Institute for Molecular Medicine Finland, FIMM, University of Helsinki, 00014, Helsinki, Finland

* Corresponding author

SARS-CoV-2 and its vaccine/immune-escaping variants continue to pose a serious threat to public health due to a paucity of effective, rapidly deployable, and widely available treatments. Here we address these challenges by combining Pegasys (IFNa) and nafamostat to effectively suppress SARS-CoV-2 infection in cell culture and hamsters. Our results indicate that Serpin E1 is an important mediator of the antiviral activity of IFNa and that both Serpin E1 and camostat can target the same cellular factor TMPRSS2, which plays a critical role in viral replication. The low doses of the drugs in combination may have several clinical advantages, including fewer adverse events and improved patient outcome. Thus, our study may provide a proactive solution for the ongoing pandemic and potential future coronavirus outbreaks, which is still urgently required in many parts of the world.

Introduction

Over the past 10 years, there have been four major viral epidemics/pandemics for which the world was unprepared. The current pandemic concerns the SARS-CoV-2 virus, which has infected over 275 million people globally, killed over 3,7 million, devastated economies, and caused other unfathomable hardships. The challenge addressed in this study is a tragic lack of effective antiviral drugs that can be deployed to treat SARS-CoV-2 infection.

So far, many monotherapies have been tested, but all have been shown to be ineffective or had limited efficacy against COVID-19 [1]. By contrast, combinational therapies are emerging as a useful tool to treat SARS-CoV-2 infection [2]. Synergistic combinational therapies can achieve better efficacy while requiring lower dosage compared to monotherapies, therefore, inducing fewer and milder adverse effects. Additionally, antiviral combinations could target emerging SARS-CoV-2 variants and prevent the development of strains resistant to monotherapies.

Our recent studies have highlighted the synergism of several compounds against SARS-CoV-2 in human lung epithelial Calu-3 cells, with IFNa-remdesivir and camostat-remdesivir combinations having the highest synergy scores in vitro [2-4]. In these studies, we were also able to show that camostat-remdesivir was effective against SARS-CoV-2 infection in human lung organoids, as well as that IFNa-remdesivir was effective in both human lung organoids and Syrian hamsters [3, 4]. Furthermore, other studies have shown that the combinations of IFNa with lopinavir–ritonavir-ribavirin, and combination of nafamostat with favipiravir are effective for treatment of patients infected with SARS-CoV-2 [5, 6].

Nafamostat, a structural analogue of camostat, is a repurposed drug which was originally approved as a short-acting anticoagulant and is also used for the treatment of pancreatitis [7]. It is currently in clinical trials for treatment of COVID-19 (NCT04623021, NCT04473053, NCT04390594, NCT04483960). IFNas and its pegylated forms are also repurposed drugs which have been shown to be effective for COVID-19 patients [8, 9]. Because previous studies have demonstrated the high therapeutic potential of nafamostat and IFNa as antiviral treatments, we hypothesized that development of IFNa-nafamostat combination may lead to practical therapeutic option against SARS-CoV-2 infection.

Results and discussion

To test our hypothesis, we first analyzed toxicity and efficacy of Pegasys (pegylated IFNa) and nafamostat, against mCherry-expressing SARS-CoV-2 [10] in human lung epithelial Calu-3 cells using fluorescence and cell

viability assay as readouts as described previously [4]. We observed that both Pegasys and nafamostat reduced SARS-CoV-2-mediated mCherry expression and rescued cells from virus-mediated death (Fig. 1a). Interestingly, Pegasys reduced SARS-CoV-2 replication less efficiently than its non-pegylated analogue, whereas nafamostat reduced SARS-CoV-2 replication more efficiently than camostat.

Second, we examined whether Pegasys-nafamostat can inhibit SARS-CoV-2 infection and protect Calu-3 cells from virus-mediated death more efficiently and at lower concentrations than monotherapies. We tested the antiviral efficacy and toxicity of the combination by monitoring SARS-CoV-2-mediated mCherry expression and cell viability. The drug combination was tested in a 6×6 dose-response matrix, where 5 doses of each combination component are combined in a pairwise manner. As a result, we obtained dose-response matrices demonstrating virus inhibition and cell viability (Fig. 1b). We plotted synergy distribution maps, showing synergy (higher than expected effect) at each pairwise dose. We calculated average ZIP synergy scores for the whole 6×6 dose-response matrices and for most synergistic 3×3 dose-regions, summarizing combination synergies into single metrics. We observed strong synergy of the Pegasys-nafamostat combination (ZIP synergy scores: 4.8 (mCherry) and 27.4 (CTG); most synergistic area scores: 13.6 (mCherry) and 36.4 (CTG)). This strong synergy indicates that, both components could be combined in vitro at decreased concentrations to achieve antiviral efficacy comparable to those of individual drugs at high concentrations.

Next, we examined whether Pegasys-nafamostat can affect the replication of SARS-CoV-2 *in vivo*. Four groups of 6 six-week-old female Syrian hamsters were injected IP with 40 µg/kg Pegasys, 10 mg/kg nafamostat, Pegasys-nafamostat combination or vehicle at day 0, 1 and 2 of infection. After 2 h of drug treatment at day 0, animals received SARS-CoV-2 strain Slovakia/SK-BMC5/2020 intranasally (10^5 pfu $TCID_{50}$ per animal). Control group of 5 hamsters remained untreated and uninfected. After 3 days, animals were anesthetized and euthanized, and the lungs were collected. Total RNA was extracted and expression of viral ORF1ab was analyzed using RT-qPCR as described previously [4]. We found that the drug combination attenuated synthesis of viral RNA more efficiently than the individual agents (Fig. 1c) and that the antiviral effect of the combination was additive *in vivo* (synergy score: 5.17), suggesting that this cocktail has high translatability.

Our recent transcriptomics analysis revealed that IFNa activates transcription of many genes including endothelial plasminogen activator inhibitor (*SERPINE1)* in Calu-3 cells and human lung organoids [4]. Serpin E1 inhibits urokinase-type and tissue-type plasminogen activators (uPA and tPA) as well as various membrane-anchored serine proteases including transmembrane protease serine 2 (TMPRSS2) [11]. Nafamostat also inhibits

TMPRSS2 [12]. Therefore, we hypothesized, that the synergy of the Pegasys-nafamostat could be achieved because both drugs target the same host factor, TMPRSS2 (Fig. 1d). To investigate this, we treated a group of 6 hamsters with an inhibitor of Serpin E1, tiplaxtinin (3 mg/kg, PO), Pegasys (40 µg/kg, IP), and nafamostat (10 mg/kg, IP) at day 0, 1 and 2 of infection. After 2 h of drug treatment at day 0, animals received SARS-CoV-2 intranasally. After 3 days, animals were anesthetized and euthanized, and the lungs were collected. Total RNA was extracted and expression of viral ORF1ab was analyzed using RT-qPCR (Fig. 1c). Tiplaxtinin treatment restored viral RNA synthesis to the level of nafamostat alone, and therefore wholly eliminated the effect of Pegasys. These results indicate that Serpin E1 is an essential mediator of IFNa activity and that TMPRSS2 can be targeted by several drugs to synergistically suppress viral infection.

It was shown that nafamostat inhibits hypercoagulopathy associated with severe COVID-19 [7]. By contrast, IFNa-induced Serpin E1 is a risk factor for thrombosis [13]. Thus, it is highly likely that the anticoagulative properties of nafamostat could compensate for the adverse effects of IFNa-induced Serpin E1 when administered in combination with each other. Moreover, combination therapy containing lower doses of Pegasys and nafamostat may reduce the likelihood of developing other side effects entirely [14], and thus be useful in treating COVID-19 patients. In addition, the Pegasys-nafamostat combination could be delivered through different administration routes, leading to greater ease of treatment. We believe further development of combination of two prescription drugs, Pegasys and nafamostat, can lead to practical therapeutic options against many viruses, for which replication depends on TMPRSS2 (e.g., CoVs and influenza viruses) [15]. Furthermore, our demonstrated proof-of-concept here shows that research on potential synergistic antiviral combinations can have significant global impact, by increasing protection of the general population against emerging and re-emerging viral diseases and filling the time between virus identification and vaccine development with life-saving countermeasures.

Materials and methods

Drugs, viruses, cells, and hamsters

Pegasys (cat. # EMEA/H/C/000395, 008767) was purchased from local pharmacy in syringes for subcutaneous injection (135µg IFNa2a/0.5mL each). Lyophilized IFNa2a (cat. # 11343504; ImmunoTools) was dissolved in sterile deionized water to obtain 200 µg/mL concentrations. Camostat mesylate (cat. # 16018, Cayman Chemicals), nafamostat mesyate (cat. # 14837, Cayman

Chemicals) and tiplaxtinin (cat. # 393105-53-8, MCE) were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) to obtain 10 mM stock solutions.

Recombinant mCherry-expressing SARS-CoV-2 (SARS-CoV-2-mCherry), and wild type human SARS-CoV-2 strains were provided by Prof. Andres Merits or the European Virus Archive global (EVAg) and propagated in Vero E6 or Vero E6/TMPRSS2 cells. To quantitate the production of infectious virions, we titered the viruses using plaque assays or ELISA.

The propagation of human non-small cell lung cancer Calu-3 have been described in our previous studies [4, 16].

Thirty-five 6-week-old healthy female Syrian hamsters were obtained from Janvier Labs. The animals were maintained in pathogen free health status according to the FELASA guidelines. The animals were individually identified and were maintained in housing rooms under controlled environmental conditions: temperature: 21 ± 2°C, humidity 55 ± 10%, photoperiod (12h light/12h dark), H14 filtered air, minimum of 12 air exchanges per hour with no recirculation. Each cage was labeled with a specific code. Animal enclosures provided sterile and adequate space with bedding material, food and water, environmental and social enrichment (group housing) as described below: IsoRat900N biocontainment system (Techniplast, France), poplar bedding (Select fine, Safe, France), A04 SP-10 diet (Safe, France), tap water, environmental enrichment, tunnel, wood sticks. Animal housing and experimental procedures were conducted according to the French and European Regulations and the National Research Council Guide for the Care and Use of Laboratory Animals. The animal BSL3 facility is authorized by the French authorities (Agreement N° D92-032-02). All animal procedures (including surgery, anesthesia, and euthanasia as applicable) were approved by the Institutional Animal Care and Use Committee of CEA and French authorities (CETEA DSV – n° 44).

Drug Testing and Drug Sensitivity Quantification

Approximately 4×10^4 Calu-3 cells were seeded per well in 96-well plates. The cells were grown for 24 h in DMEM-F12, supplemented with 10% FBS and Pen–Strep. The medium was then replaced with DMEM-F12 containing 0.2% BSA, Pen–Strep and the compounds in 3-fold dilutions at 7 different concentrations. No compounds were added to the control wells. The cells were infected with SARS-CoV-2-mCherry strains at a moi of 0.1 or mock. After 48h drug efficacy on SARS-CoV-2-mCherry infected cells was measured on PFA- or acetone-fixed cells with fluorescence. After 72 h of infection, a CellTiter-Glo (CTG) assay was performed to measure cell viability.

The half-maximal cytotoxic concentration (CC_{50}) for each compound was calculated based on viability/death curves obtained on mock-infected cells after non-linear regression analysis with a variable slope using GraphPad Prism software version 7.0a. The half-maximal effective concentrations (EC_{50}) were calculated based on the analysis of the viability of infected cells by fitting drug dose-response curves using four-parameter (*4PL*) logistic function *f(x)*:

$$f(x) = A_{min} + \frac{A_{max} - A_{min}}{1 + (\frac{x}{m})^{\lambda}}, \quad (1)$$

where *f(x)* is a response value at dose *x*, A_{min} and A_{max} are the upper and lower asymptotes (minimal and maximal drug effects), *m* is the dose that produces the half-maximal effect (EC_{50} or CC_{50}), and λ is the steepness (slope) of the curve. The relative effectiveness of the drug was defined as selectivity index ($SI = CC_{50}/EC_{50}$).

Drug Combination Testing and Synergy Calculations

Calu-3 cells were treated with different concentrations of two drugs and infected with SARS-CoV-2-mCherry (moi 0.1) or mock. IAfter 48 h, cell viability and reporter protein expression were measured. To test whether the drug combinations act synergistically, the observed responses were compared with expected combination responses. The expected responses were calculated based on the ZIP reference model using SynergyFinder version 2 [17, 18]. Final synergy scores were quantified as average excess response due to drug interactions (i.e., 10% of cell survival beyond the expected additivity between single drugs represents a synergy score of 10). Additionally, we calculated most

synergistic area scores for each drug combination – the most synergistic 3-by-3 dose-window in dose-response matrixes.

Prophylactic Study of Remdesivir, IFNaa and Their Combination Against SARS-CoV-2 Infection in Hamsters

Thirty-five animals were weighed and divided into 6 homogenous groups of 5-6 animals. Group 1 (5 non-infected controls) remained unmanipulated. Group 2 received vehicle (10mL/kg) 3 times by IP route on Day 0 (t-2h), Day 1 and Day 2. Group 3 received nafamostat (10mg/kg) 3 times by IP route on Day 0 (t-2h), Day 1 and Day 2. Group 4 received Pegasys (40 μg/kg) 3 times by IP route on Day 0 (t-2h), Day 1 and Day 2. Group 5 received a mixture of nafamostat and Pegasys 3 times by IP route on Day 0 (t-2h), Day 1 and Day 2. Group 6 received a mixture of the nafamostat and Pegasys 3 times by IP route on Day 0 (t-2h), Day 1 and Day 2 and Tiplaxtinin (3mg/kg) 3 times by PO route on Day 0 (t-2h), Day 1 and Day 2. Groups 2-6 received SARS-CoV-2 intranasally. Animal viability, behavior and clinical parameters were monitored daily. After 3 days animals were deeply anesthetized using a cocktail of 30 mg/kg (0.6 mL/kg) Zoletil and 10 mg/kg (0.5 mL/kg) Xylazine IP. Cervical dislocation followed by thoracotomy was performed before lung collection. The entire left lungs and superior, middle, post-caval and inferior lobes of right lungs were put in RNAlater tissue storage reagent overnight at 4°C, then stored at -80°C until RNA extraction.

RT-qPCR Analysis

Viral RNA was extracted using the QIAamp Viral RNA Mini Kit (Qiagen). RT-PCR was performed using SuperScript™ III One-Step qRT-PCR System kit (commercial kit #1732-020, Life Technologies) with primers ORF1ab_Fw: CCGCAAGGTTCTTCTTCGTAAG, ORF1ab_Rv: TGCTATGTTTAGTGTTCCAGTTTTC, ORF1ab_probe: Hex-AAGGATCAGTGCCAAGCTCGTCGCC-BHQ-1 targeting a region on ORF1ab. RT-qPCR was performed using a Bio-Rad CFX384™and adjoining software. The relative gene expression differences were calculated using β-Actin as control and the results were represented as relative units (RU). Technical triplicates of each sample were performed on the same qPCR plate and non-templates and

non-reverse transcriptase samples were analysed as negative controls. Statistical significance ($p < 0.05$) of the quantitation results was evaluated with t-test. Benjamini-Hochberg method was used to adjust the p-values.

Ethics approval and consent to participate: Standard operational procedures were approved by institutional safety committee.

Consent for publication: All authors have read and agreed to the published version of the manuscript.

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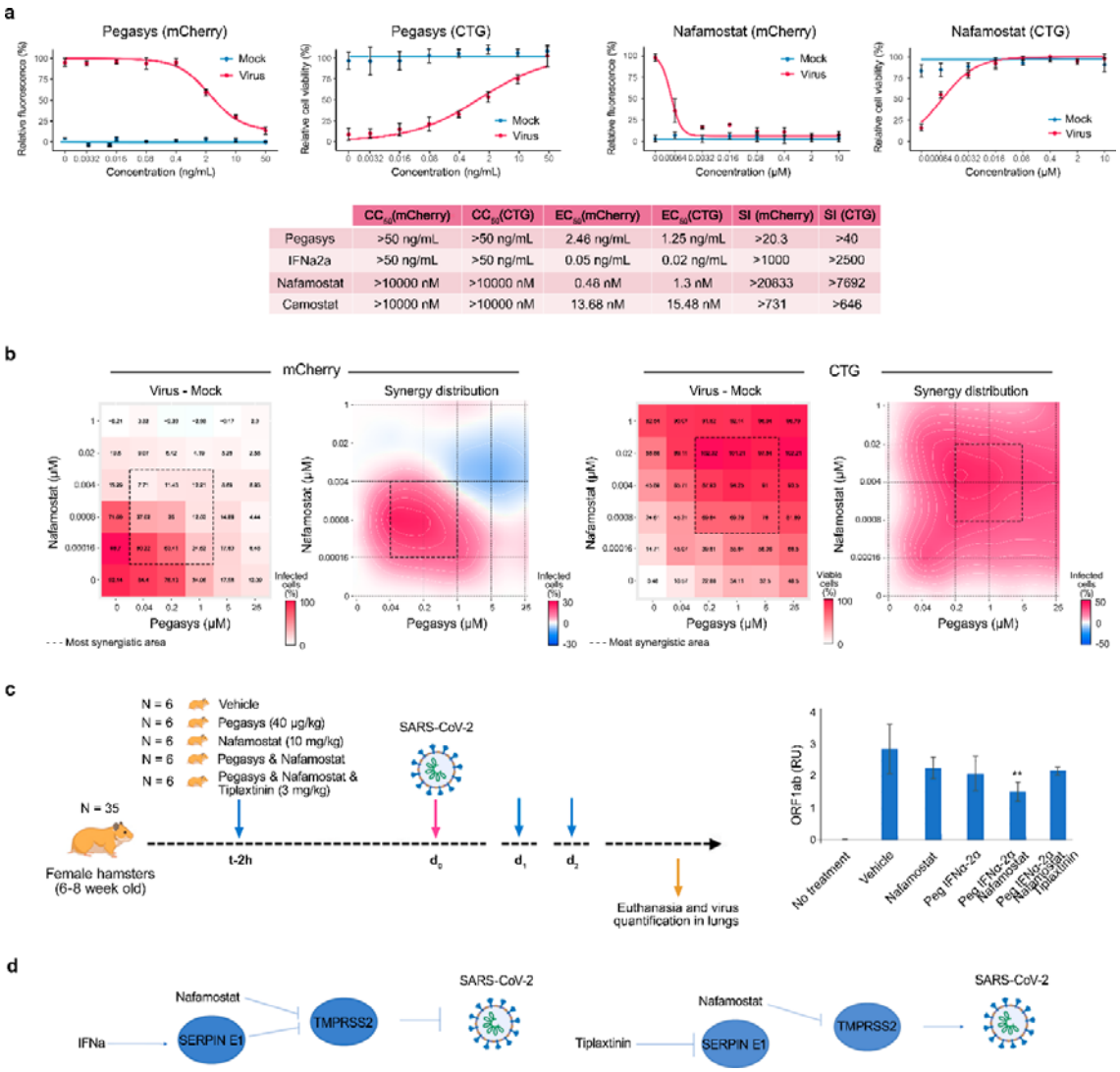

	CC_{50}(mCherry)	CC_{50}(CTG)	EC_{50}(mCherry)	EC_{50}(CTG)	SI (mCherry)	SI (CTG)
Pegasys	>50 ng/mL	>50 ng/mL	2.46 ng/mL	1.25 ng/mL	>20.3	>40
IFNa2a	>50 ng/mL	>50 ng/mL	0.05 ng/mL	0.02 ng/mL	>1000	>2500
Nafamostat	>10000 nM	>10000 nM	0.46 nM	1.3 nM	>20833	>7692
Camostat	>10000 nM	>10000 nM	13.68 nM	15.48 nM	>731	>646

Figure 1. Nafamostat-interferon-alpha combination suppresses SARS-CoV-2 infection in vitro and in vivo.

(**a**) Pegasys and nafamostat attenuate virus-mediated reporter protein expression and rescue Calu-3 cells from SARS-CoV-2-mediated death. Calu-3 cells were treated with increasing concentrations of IFNa2a or nafamostat and infected with the SARS-CoV-2-mCherry or mock. After 48 h, the virus-mediated mCherry expression was measured (red curves). After 72 h, viability of virus- and mock-infected cells was determined using a CTG assay (yellow and blue curves, respectively). Mean ± SD; n = 3. Toxicity and anti-SARS-CoV-2 activity of Pegasys and nafamostat was quantified and compared to that of IFNa2a and camostat.

(**b**) Pegasys and nafamostat combination act synergistically against SARS-CoV-2-mCherry infection in Calu-3 cells. Calu-3 cells were treated with increasing concentrations of IFNa2a, nafamostat or both drugs and infected with the SARS-CoV-2-mCherry or mock. After 48 h, the virus-mediated mCherry expression was measured (red curves). After 72 h, viability of virus- and mock-infected cells was determined using a CTG assay (yellow and

blue curves, respectively). The 6 × 6 dose–response matrices and interaction landscapes the drug combination was obtained using fluorescence analysis as well as cell viability assay (CTG) on mock-, and SARS-CoV-2-mCherry-infected Calu-3 cells. ZIP synergy score was calculated for the drug combinations. The selectivity for the indicated drug concentrations was calculated (selectivity = efficacy-(100-Toxicity)). ZIP synergy scores were calculated for indicated drug combinations.

(**c**) Pegasys-nafamostat combination acted synergistically against wild-type SARS-CoV-2 in Syrian hamsters, whereas tiplaxtinin wholly eliminated the effect of Pegasys. Schematic representation of the experimental setup (left panel). RT-qPCR analysis of selected viral RNA (right panel). Expression of viral RNA was normalized to b-actin control. Mean ± SD, n = 6 ($n_{control\ group}$=5). Statistically significant differences in viral gene expression between non-treated and treated animals are indicated with asterisks (**p<0.05, *p<0.1, Wilcoxon test).

(**d**) Schematic representation of synergistic mechanism of action of Pegasys and nafamostat, as well as the effect of tiplaxtinin on Pegasys-induced Serin E1.