

Modular Nanotransporters Capable of Causing Intracellular Degradation of the N-Protein of the SARS-CoV-2 Virus in A549 Cells with Temporary Expression of This Protein Fused with a Fluorescent Protein mRuby3

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Abstract—Modular nanotransporters (MNTs) containing an antibody-like molecule, monobody, to the N-protein of the SARS-CoV-2 virus, as well as an amino acid sequence that recruits the Keap1 E3 ligase (E3BP) were created. This MNT also included a site for cleavage of the E3BP monobody from the MNT in acidic endocytic compartments. It was shown that this cleavage by the endosomal protease cathepsin B leads to a 2.7-fold increase in the affinity of the E3BP monobody for the N-protein. Using A549 cells with transient expression of the N-protein fused with the fluorescent protein mRuby3, it was shown that incubation with MNT leads to a significant decrease in mRuby3 fluorescence. It is assumed that the developed MNTs can serve as a basis for the creation of new antiviral drugs against the SARS-CoV-2 virus.

Keywords: nucleocapsid protein, SARS-CoV-2, flow cytometry, thermophoresis, cathepsin B, E3 ligase, Keap1

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The need for the development of new antiviral drugs has been particularly clearly demonstrated by the SARS-CoV-2 coronavirus pandemic. The most commonly used drugs are low-molecular-weight inhibitors of viral activity [1]. However, they cannot be selected for all protein targets, unlike antibody-like molecules, which can be generated to nearly any protein antigen [2, 3]. Not surprisingly, various antibody-like molecules are increasingly used in various delivery systems for bioactive molecules [4]. In the case of the SARS-CoV-2 virus, the nucleocapsid protein (or N-protein), which is essential for the viral capsid assembly, can be used as the target protein [5–7]. A previously obtained antibody-like molecule, monobody (NC2), has a high affinity for this protein [8]. Previously, we developed modular nanotransporters (MNTs) containing this monobody and capable of binding to the N-protein in target cells [9], referred to in this work as MNT₀. The next MNT variant, MNT₁, differed from MNT₀ in that it additionally contained the DPETGEYL amino acid sequence (hereinafter referred to as E3BP), which is capable of high-affinity

binding to the Keap1 ubiquitin ligase [10]. We assumed that the binding of MNT₁ simultaneously to Keap1 and the N-protein would lead to the N-protein degradation as a result of its ubiquitination [11]. MNT₁, similarly to MNT₀, contained a site for cleavage of the E3BP monobody from MNT₁ in acidified endocytic compartments. In this work, we studied both the interaction of MNT₁ and cleaved MNT₁ with N-protein in solution and the effect of MNT₁ on the N-protein content in A549 cells that temporarily express this protein.

The production and purification of the N-protein and MNT₀ were performed as described in [9]. From the MNT described previously [9], using site-specific mutagenesis, we derived a gene encoding the MNT affibody(EGFR)-HisTag-DTox-HMP-FKFL-E3BP-NC2. In this MNT, affibody(EGFR) is the affibody to the epidermal growth factor receptor, DTox is the translocation domain of diphtheria toxin, HMP is the hemoglobin-like protein of *E. coli*, FKFL is the cleavage site for the endosomal protease cathepsin B, and E3BP is the amino acid sequence that recruits the Keap1 E3 ligase and NC2 monobody to the N-protein. MNT₁ was expressed in the *E. coli* strain Ros(DE3)pLysS. MNT₁ expression was auto-induced by incubation at 18°C for 48 h. MNT₁ was isolated from the soluble fraction [12] and then purified by

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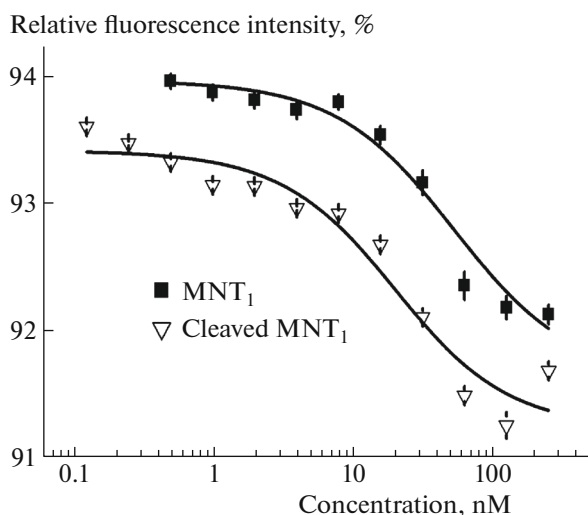


Fig. 1. Dependences of the relative fluorescence intensity 20 s after the start of thermophoresis on the concentration of MNT₁ or cleaved MNT₁ at a constant concentration of N-protein labeled with AF488 (5 nM). The fluorescence intensity before the start of thermophoresis was taken as 100%. The standard error of relative fluorescence intensity determination is indicated (14–17 replicates).

affinity chromatography on HisTrap FF column (Cytiva). Denaturing polyacrylamide gel electrophoresis demonstrated a sufficient degree of purity of the obtained MNT₁ (86.6%).

To obtain cleaved MNT₁, 4 μ M MNT₁ was incubated with 4 μ g/mL activated cathepsin B (Native human Cathepsin B protein (ab90387, Abcam)). Cathepsin B was activated as described in [14]. MNT₁ was cleaved at pH 5.5 in the presence of 0.001% SDS to prevent MNT₁ aggregation. The interaction of MNT₁ and cleaved MNT₁ with the N-protein was studied as described in [13] using thermophoresis on a Monolith NT.115 Series instrument (NanoTemper Technologies GmbH, Germany) in a buffer containing 10 mM NaH₂PO₄ and 150 mM NaCl (pH 8.0). N-protein was labeled with the fluorescent dye AF488 as described in [13] with the same degree of modification. Transfection of A549 cells with a plasmid (3–4% of transfected cells) encoding an N-protein fused with the fluorescent protein mRuby3 was performed according to [13].

At a fixed concentration of AF488-labeled N-protein (5 nM), thermophoresis was used to obtain dependences of relative fluorescence intensity 20 s after the start of thermophoresis on the concentration of MNT₁ (Fig. 1, squares) or cleaved MNT₁ (Fig. 1, open triangles). The fluorescence intensity before the start of thermophoresis was taken as 100%. For each experiment, three to four such curves were obtained, and the entire experiment was performed in three or four replicates. From each curve, the dissociation constant of the complex of MNT₁ or cleaved MNT₁ with

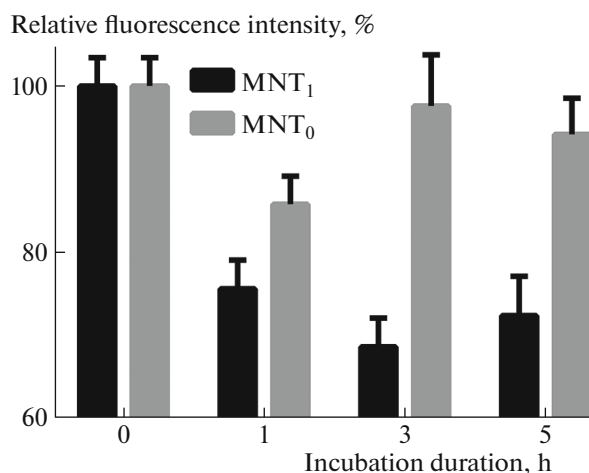


Fig. 2. Relative fluorescence intensity of A549 cells incubated for different times with 500 nM MNT₁ or 500 nM MNT₀. The fluorescence intensity of cells to which MNT was not added was taken as 100%. The mean values with the corresponding standard error are shown ($n = 3–9$).

N-protein was determined. It was averaged over all 12–17 curves, and the relative measurement error was determined. The dissociation constants of the complexes of MNT₁ or cleaved MNT₁ with N-protein were 47 ± 3 and 17 ± 4 nM, respectively. For the cleaved MNT₁, this value is close to the constant for the free NC2 monobody (6.7 nM [8]). Thus, the cleavage of MNT₁ by cathepsin B results in an approximately threefold increase in its affinity for the N-protein.

The process of degradation of the N-mRuby3 fusion protein in A549 cells temporarily expressing this protein was monitored using flow cytometry with a MACSQuant Analyzer (Miltenyi Biotec, France) in the fluorescence channel 571–601 nm; fluorescence was excited using a laser with a wavelength of 561 nm. Cells transiently expressing the N-mRuby3 protein were incubated with 500 nM MNT₁ or MNT₀ for a specified time, washed, removed from the substrate, and studied using a flow cytometer in the mRuby3 fluorescence channel. Figure 2 shows the dependence of A549 cell fluorescence on the duration of incubation with MNT₁ or MNT₀. After 5-h incubation, the average fluorescence intensity of A549 cells was significantly ($p < 0.05$, Mann–Whitney U test) lower when the cells were incubated with MNT₁ as compared to the incubation with MNT₀. The decrease in the mRuby3 fluorescence intensity can be attributed to the degradation of the N-mRuby3 fusion protein. The data obtained allow us to conclude that the insertion of the amino acid sequence that recruits the Keap1 E3 ligase into the MNTs developed by us can lead to degradation of the N-protein in target cells.

As we showed earlier, the cleavage of the monobody from MNT₀ leads to a significant decrease in the dissociation constant of the complex of the N-protein

with this monobody (from 116 ± 20 to 10 ± 3 nM) [9]. In this work, a noticeable increase in affinity was also observed for MNT₁ (the dissociation constant decreased from 47 ± 3 to 17 ± 4 nM). In other words, the cleaved fragment will interact with the N-protein in the target cell much better than the full-length MNT. It is known that competitive interaction with the N-protein can potentially disrupt the entire process of assembly of new viral particles [15] and, therefore, suppress the spread of the virus in the body. In this study, this effect on the N-protein is also accompanied by its probable degradation due to the recruitment of the E3 ligase to it, which, in our opinion, should radically affect the viral capsid assembly.

We assume that the developed MNTs can serve as a basis for creating new antiviral drugs against the SARS-CoV-2 virus. Moreover, in the future, the approach used in this work may be effective not only against this virus, but also against other viruses.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This work does not contain any studies involving human and animal subjects.

CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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