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Article

Molecular Aggregation Strategy for Inhibiting DNases

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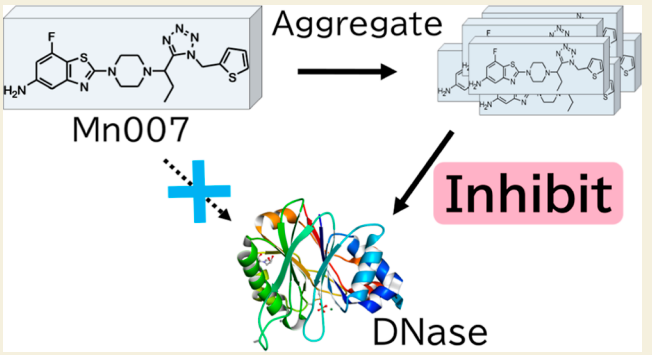
ABSTRACT: This study highlights the novel potential of molecular aggregates as inhibitors of a disease-related protein. Enzyme inhibitors have been studied and developed as molecularly targeted drugs and have been applied for cancer, autoimmune diseases, and infections. In many cases, enzyme inhibitors that are used for therapeutic applications interact directly with enzymes in a molecule-to-molecule manner. We found that the aggregates of a small compound, Mn007, inhibited bovine pancreatic DNase I. Once Mn007 molecules formed aggregates, they exhibited inhibitory effects specific to DNases that require divalent metal ions. A DNase secreted by *Streptococcus pyogenes* causes streptococcal toxic shock syndrome (STSS). STSS is a severe infectious disease with a fatality rate exceeding 30% in patients, even in this century. *S. pyogenes* disrupts the human barrier system against microbial infections through the secreted DNase. Until now, the discovery/development of a DNase inhibitor has been challenging. Mn007 aggregates were found to inhibit the DNase secreted by *S. pyogenes*, which led to the successful suppression of *S. pyogenes* growth in human whole blood. To date, molecular aggregation has been outside the scope of drug discovery. The present study suggests that molecular aggregation is a vast area to be explored for drug discovery and development because aggregates of small-molecule compounds can inhibit disease-related enzymes.



KEYWORDS: *deoxyribonuclease, enzyme inhibitor, neutrophil extracellular traps, molecular aggregation, Streptococcus pyogenes*

INTRODUCTION

Enzymatic reactions are fundamental to biological functions. Typically, enzymes interact with their substrates with high specificity. There are several modes of enzyme inhibition, including competitive, noncompetitive, and uncompetitive inhibition. In many cases, inhibitors that are used for therapeutic applications interact directly with enzymes in a molecule-to-molecule manner. There are only a few reports of molecular aggregates that inhibit enzymes in aqueous solutions.[1−3] Such inhibitors are thought to prevent enzymes from accessing their substrates via physical adsorption and are considered nonspecific to enzymes.[4] Therefore, they are deemed unsuitable for therapeutic applications that target specific enzymes in vivo. This means that if the aggregate of small compounds specifically inhibits a target enzyme, molecular aggregates can create a new modality in pharmaceutical development.

*Streptococcus pyogenes*is a group A *Streptococcus* that causes streptococcal toxic shock syndrome (STSS).[5,6] STSS is a severe infectious disease with a fatality rate exceeding 30% in patients, even in this century.[7,8] Owing to the rapid spread of the lesions, the primary treatment involves extensive surgical excision around the infected area, followed by broad systemic administration of antibiotics. These treatments place a significant burden on the patient.

Humans have various defense systems against microbial infections. One of these is neutrophil extracellular traps (NETs), which consist of neutrophil genome DNA. *S. pyogenes* secretes a DNA-degrading enzyme (DNase) that degrades NETs and impairs the defense system, allowing for aggressive invasion of the body. DNase is therefore being investigated as a potential molecular target for treating STSS.[9−11]

Despite the importance of DNase inhibition, there are a limited number of reports on DNase inhibitors.[12,13] EDTA,[14] crystal violet,[15] and actin[16] have been reported to inhibit bovine pancreatic DNase I. However, EDTA and crystal violet show cytotoxicity, and actin is derived from animals, which suggests a potential risk in therapeutic application.

In this study, we report that the aggregates of a small molecule, Mannan 007 (Mn007), specifically inhibit the DNases. Mn007 was previously identified as a potential lead compound for treating Fukuyama-type congenital muscular

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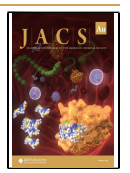

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dystrophy (FCMD).[17,18] In the course of elucidating the therapeutic mechanism of Mn007 against FCMD, we discovered that the aggregates of Mn007 inhibited bovine pancreatic DNase I (Figure 1a). Thus, we examined the

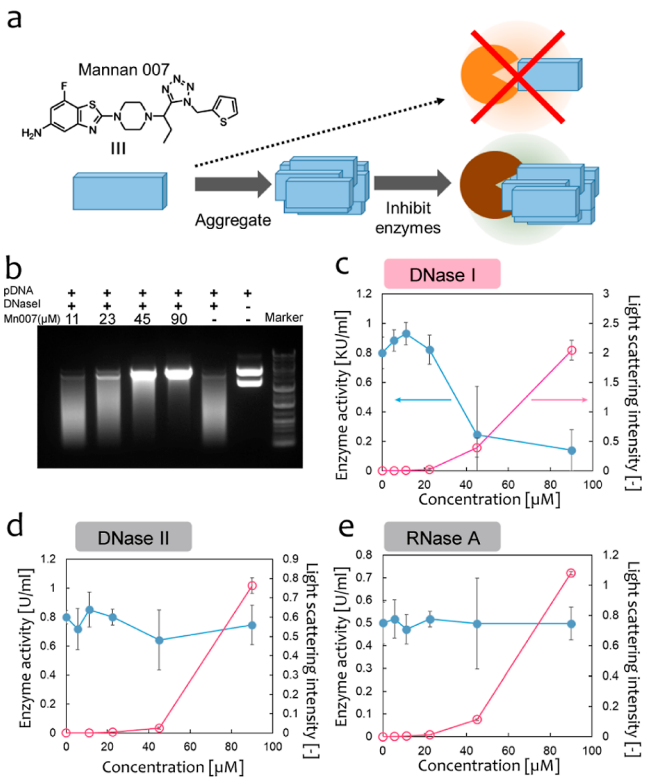

Figure 1. (a) Schematic illustration of Mn007 aggregation and subsequent enzyme inhibition. (b) Inhibition of DNase I by Mn007. The degradation of pDNA by DNase I was observed by 2% agarose gel electrophoresis. (c) Concentration-dependent aggregation and DNase I inhibition by Mn007. (d) DNase II assay and (e) RNase A assay as Mn007 aggregates in the reaction solutions. DNase I and DNase II activities were measured by tracking the degradation of FITC-modified DNA. RNase A activity was measured using TAMRA-modified RNA. Aggregation of Mn007 was assessed by measuring the light-scattering intensity of the enzyme reaction solution. The data values represent the mean ± SD of three independent experiments.

enzyme inhibition by the aggregates of Mn007 in detail and found that this inhibition was specific to DNase. Moreover, we explored the potential of Mn007 to act as a therapeutic agent for STSS.

RESULTS AND DISCUSSION

Inhibition of DNase I by Mn007 Aggregates

The inhibitory activity of racemic Mn007 was evaluated using bovine pancreatic DNase I. The degradation of plasmid DNA (pDNA) by DNase I was observed using agarose gel electrophoresis. A Tris−HCl buffer (100 mM; pH 7.5) containing Mg^{2+} ions (6 mM) was used as the reaction buffer. In the absence of Mn007, smear bands resulting from the degradation of the pDNA were observed. The addition of Mn007 reduced the smear bands, and intrinsic bands for pDNA were observed at 45 and 90 μM Mn007 (Figure 1b; for a detailed explanation, see Figure S2). These results imply that Mn007 inhibited DNase I in a concentration-dependent manner.

The inhibitory effect of Mn007 on DNase I was quantitatively examined using fluorescently labeled oligoDNA. An oligoDNA (20-mer) modified with FITC at the 5′ end (FITC-DNA) and its complementary strand with an extra guanine sequence at the 3′ end were prepared (Supporting Information). FITC-DNA was hybridized with the complement, leading to the quenching of FITC due to its interaction with the guanine residues.[19,20] When DNase I cleaved this double-stranded DNA (dsDNA), the fluorescence of FITC recovered with the reaction time. The activity of DNase I was evaluated by measuring the fluorescence of FITC over time. This DNase I assay also indicated the inhibition of DNase I by Mn007 (Figure 1c), which was consistent with the gel electrophoresis experiments (Figure 1b). Mn007 began to inhibit DNase I (0.1 μg/mL) at concentrations higher than 23 μM and completely inhibited DNase I at 90 μM. The 50% inhibitory concentration (IC_{50}) of Mn007 was calculated to be 45 μM.

When the reaction solution contained more than 45 μM Mn007, it was turbid. Therefore, we hypothesized that the inhibitory activity of Mn007 on DNase I was associated with the aggregation state of Mn007. The aggregation of Mn007 in the solution was detected by using light-scattering measurements. The light-scattering intensity of the solution increased at Mn007 concentrations higher than 23 μM (Figure 1c). The exact critical aggregation concentration (CAC) of Mn007 was calculated to be 42.5 μM (Figure S5a). These results indicate that DNase inhibition by Mn007 began at the CMC of Mn007.

The inhibition of enzymes other than DNase I by Mn007 was also investigated. DNase II and RNase A are nucleic acid-degrading enzymes that do not require divalent metal ions. In the reaction solution containing DNase II, Mn007 began to aggregate at 45 μM (Figure 1d). Even at concentrations above the CAC, Mn007 did not inhibit DNase II (Figure 1d). For the RNase A assay, an oligoRNA modified with TAMRA (TAMRA-RNA, see Supporting Information) was used as a substrate. Mn007 began to aggregate at a concentration of 45 μM under the RNase reaction conditions, but no RNase A inhibition was observed. Furthermore, the inhibitory effects of Mn007 aggregates were tested for 4 different kinds of enzymes: α-mannosidase from Jack bean, which is a glycosidase whose activity depends on Zn^{2+} ions; β-glucosidase from sweet almond, which is a glycosidase without metal ions as a cofactor; horseradish peroxidase that involves Fe(III) and Fe(IV) as a redox cofactor; and glucose oxidase, which is a glucose oxidizing enzyme with flavinadenine dinucleotide as a redox cofactor. Mn007 aggregates did not inhibit these enzymes at all (Figure S3). These results suggest that the aggregate of Mn007 was a specific inhibitor of DNase I. Unlike previously reported inhibitors that exerted their function by aggregation and lacked enzyme specificity, the aggregate of Mn007 showed inhibiting activity specific to DNase I.

Mechanism of DNase I Inhibition

The importance of molecular aggregation was further investigated. 2-Hydroxypropyl-β-cyclodextrin (HyβCD) was used as a solubilizing agent for Mn007. While the DNase I assay solution containing 90 μM Mn007 was turbid, the addition of HyβCD (100 mM) immediately turned the solution transparent (Figure 2a). This suggests that HyβCD solubilized Mn007 above its CAC, likely through the formation of an inclusion complex. At various concentrations of HyβCD, the inhibitory effect of Mn007 on DNase I was examined. As

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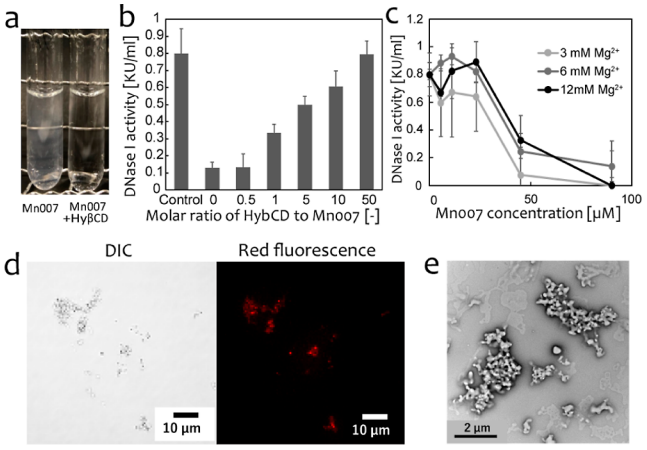

Figure 2. (a) Appearance of the reaction solutions containing 90 μM Mn007 and 100 mM HyβCD. (b) Effect of the HyβCD concentration on DNase I activity in the presence of 90 μM Mn007. (c) DNase I assay with 90 μM Mn007 with varying Mg^{2+} concentration. (d) CLSM images of Mn007 aggregates in the presence of DNase I labeled with rhodamine B isothiocyanate (Rho-DNase I). DIC represents a differential interference contrast image. (e) TEM image of the aggregates of Mn007. The data values represent the mean ± SD of three independent experiments.

shown above, 90 μM Mn007 almost completely inhibited DNase I. However, as the concentration of HyβCD increased, the DNase I activity gradually resumed. Mn007 finally did not inhibit DNase I when the HyβCD concentration was 50 times greater than that of Mn007 (Figure 2b). It should be noted that the addition of HyβCD did not affect the activity of DNase I in the absence of Mn007. This observation indicated that the aggregation of Mn007 was essential for the inhibition of DNase I.

Some inhibitors inhibit enzymes by binding to cofactors rather than to the enzymes directly. Because the activity of DNase I is enhanced in the presence of divalent metal ions, EDTA acts as an inhibitor of DNase I by chelating the metal ions. We investigated the effect of the Mg^{2+} concentration on the inhibition activity of Mn007. At Mg^{2+} concentrations of 3 , 6 , and 12 mM, Mn007 inhibited DNase I at concentrations of 45 μM or higher (Figure 2c). The Mn007 concentration at which Mn007 inhibited DNase I was constant regardless of the Mg^{2+} concentration. The results suggest that the inhibition of DNase I was not induced by the interaction of Mn007 with Mg^{2+}.

DNase I modified with rhodamine B isothiocyanate (Rho-DNase I) was prepared to visualize the interaction of DNase I with Mn007 aggregates. Aggregates of Mn007 smaller than 30 μm were observed using confocal laser scanning microscopy (CLSM) (Figure 2e). Simultaneously, red fluorescence was observed on the Mn007 aggregates, suggesting the interaction of Rho-DNase I with the Mn007 aggregates. Notably, the Mn007 aggregates did not exhibit any intrinsic red fluorescence (Figure S4a).

Some small molecules and peptides bind to dsDNA.[21,22] Double-stranded FITC-DNA without fluorescence quenching was prepared and added to the Mn007 aggregates. No green fluorescence was observed for the Mn007 aggregates (Figure S4b), indicating negligible interaction between double-stranded FITC-DNA and the Mn007 aggregates. The melting temperature (T_m) of unlabeled dsDNA was analyzed in the presence of Mn007 (Figure S5). The addition of Mn007 did not alter the T_m, which also indicated that Mn007 had little interaction with dsDNA. These results suggest that the inhibition of DNase I was not due to the intercalation of Mn007 into DNA but to the interaction of DNase I and Mn007.

Transmission electron microscopy (TEM) with negative staining was employed for a topological observation of Mn007 aggregates. The aggregates exhibited a chain-like nanostructure and varied in size, showing a wide variety (Figure 2e). The aggregates had a smooth and round surface, which indicated that they are not crystals.

Mn007 Derivatives

The molecular structure of Mn007 was modified to control its aggregation properties. Five different Mn007 derivatives were synthesized by altering potential functional groups involved in the aggregation while retaining the basic skeleton of Mn007. The relationship between the DNase I inhibitory activity and the aggregation properties of the derivatives was examined (Figure 3a). Light-scattering measurements revealed that the

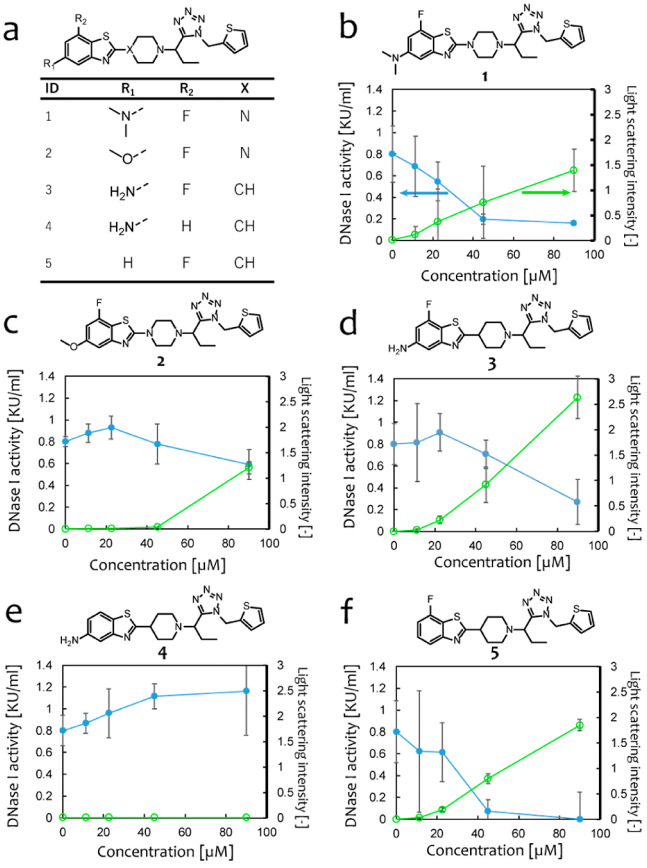

Figure 3. (a) Molecular structures of Mn007 derivatives. (b–f) DNase I inhibition assay and light-scattering measurements for Mn007 derivatives: (b) compound **1**, (c) compound **2**, (d) compound **3**, (e) compound **4**, and (f) compound **5**. The data values represent the mean ± SD of three independent experiments.

CACs of the Mn007 derivatives had the following order: **1** (16.8 μM) ≤ **5** (19.6 μM) < Mn007 (42.5 μM) ≤ **3** (46.6 μM) < **2** (90.7 μM) < **4** (177 μM) (Figure S6). In addition, the IC_{50} was determined for each compound: **1** (32 μM), **2** (136 μM), **3** (77 μM), and **5** (32 μM) (Figure 3b–f). The IC_{50} of compound **4** was not determined because compound **4** did not inhibit DNase I at 90 μM or less. The IC_{50} order of the

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JACS Au 2024, 4, 2262–2266

derivatives and Mn007 was consistent with the CAC order. These observations support the importance of aggregation in the inhibitory activity.

Potential Application in STSS Treatment

A potential therapeutic use of Mn007 was explored for STSS. *S. pyogenes* secretes DNase to degrade NETs, thereby disrupting the human defense system against microbial infections. Initially, the inhibition activity of Mn007 was assayed for DNase secreted by *S. pyogenes*. The culture supernatant of *S. pyogenes* contained DNase, which requires Mg^{2+} and Ca^{2+}, similar to DNase I.[11] When Mn007 was added to the supernatant, the DNase activity decreased proportionally to the Mn007 concentration (>the CAC) (Figure 4a). Mn007 also inhibited the DNase secreted by *S. pyogenes*.

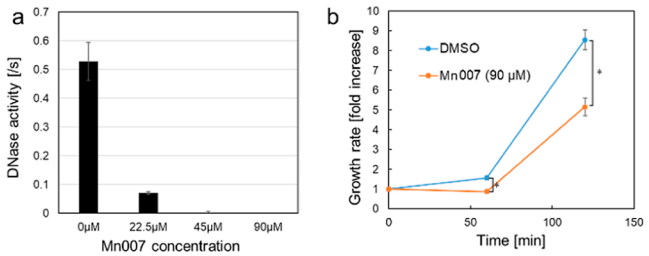

Figure 4. (a) Mn007 inhibition of the DNase secreted by *S. pyogenes*. (b) Suppression of *S. pyogenes* growth in human whole blood in the absence and presence of Mn007. The data values represent the mean ± SD of three independent experiments. *$p < 0.05$.

The inhibitory effect of Mn007 on *S. pyogenes* growth was examined by culturing *S. pyogenes* in human whole blood containing neutrophils.[23,24] After culturing for 60 min, the bacterial number increased by 56% in the absence of Mn007 (Figure 4b). However, in the presence of Mn007, it decreased by 13%. The growth of *S. pyogenes* was suppressed by Mn007, likely because Mn007 inhibited the DNase secreted by *S. pyogenes* and prevented the degradation of NETs released by neutrophils. Although after 120 min *S. pyogenes* grew in the absence and presence of Mn007, the growth rate in the presence of Mn007 was 40% lower than that in the absence of Mn007. Given the limited number of neutrophils in the blood sample, the neutrophils may have depleted over the long incubation, allowing *S. pyogenes* to return to growth.

In our previous study, Mn007 did not show cytotoxicity.[18] Mn007 would be the first case of a DNase inhibitor applied for therapeutic use. Because *S. pyogenes* infections worsen rapidly (within a few days), even temporary suppression of bacterial growth would significantly improve patient outcomes.

CONCLUSIONS

This is the first report that the molecular aggregate of a small molecule can be used as an inhibitor of a disease-related protein. We succeeded in inhibiting DNase using aggregates of Mn007 that are not cytotoxic. Various investigations demonstrated that the aggregates of Mn007 bound directly to a DNase molecule, leading to the inhibition. The inhibitory activity was specific to DNase that requires Mg^{2+} and Ca^{2+} and was not observed for other nucleic acid-degrading enzymes. Mn007 also inhibited DNase secreted by *S. pyogenes* that is essential for its infection, which led to the suppression of *S. pyogenes* growth. Molecular aggregation has not been a focal point in the development of enzyme inhibitors until now. There are several enzymes for which inhibitors have not been developed. We believe that molecular aggregation will provide a rational approach for the discovery and development of novel inhibitors for those enzymes, leading to a new strategy in drug development.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacsau.4c00210.

Materials and methods; characterization of compounds; detailed interpretation for the gel electrophoresis ; enzyme inhibition by Mn007 aggregates ; interaction between Mn007 and Rho-DNase I; melting temperature measurements; and CAC measurements (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. CRediT: **Kenta Morita** conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, validation, visualization, writing-original draft, writing-review & editing; **Tomoko Moriwaki** data curation, investigation; **Shunsuke Habe** investigation, validation; **Mariko Taniguchi-Ikeda** conceptualization, funding acquisition, investigation, methodology, project administration,

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resources, writing-original draft, writing-review & editing; **Tadao Hasegawa** data curation, investigation, methodology, resources, supervision, writing-review & editing; **Yusuke Minato** funding acquisition, supervision, writing-review & editing; **Takashi Aoi** conceptualization, data curation, funding acquisition, investigation, methodology, resources, supervision, validation, writing-review & editing; **Tatsuo Maruyama** conceptualization, data curation, funding acquisition, methodology, project administration, resources, supervision, writing-original draft, writing-review & editing.

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Notes

The authors declare no competing financial interest.

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