A Quantum Chemical Study on the Anti-SARS-CoV-2 Activity of TMPRSS2 Inhibitors

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ABSTRACT

Nafamostat and camostat are known to inhibit the spike protein-mediated fusion of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) by forming a covalent bond with the human transmembrane serine protease 2 (TMPRSS2) enzyme. Previous experiments revealed that the TMPRSS2 inhibitory activity of nafamostat surpasses that of camostat, despite their structural similarities; however, the molecular mechanism of TMPRSS2 inhibition remains elusive. Herein, we report the energy profiles of the acylation reactions of nafamostat, camostat, and a nafamostat derivative by quantum chemical calculations using a combined molecular cluster and polarizable continuum model (PCM) approach. We further discuss the physicochemical relevance of their inhibitory activity in terms of thermodynamics and kinetics. Our analysis attributes the strong inhibitory activity of nafamostat to the formation of a stable acyl intermediate and its low activation energy during acylation with TMPRSS2. The proposed approach is also promising for elucidating the molecular mechanisms of other covalent drugs. (149 words)

TOC GRAPHICS



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The outbreak of the new coronavirus (severe acute respiratory syndrome coronavirus 2; SARS-CoV-2¹) infection (COVID-19) has caused severe public health problems and its impact also changed our way of life, including our economy and culture^{2, 3}. Numerous COVID-19 treatments have been studied, resulting in the successful development of COVID-19 vaccines and anti-SARS-CoV-2 agents^{4, 5} that are gradually returning our lives to pre-pandemic conditions⁶. Meanwhile, many studies have reported effective COVID-19 treatment methods at the basic research level^{7, 8}, that are expected to be crucial in preparing for unknown viral diseases.

SARS-CoV-2 is transmitted by the binding of a spike protein (S protein) in the envelope to the angiotensin-converting enzyme 2 (ACE2) receptor on the human cell membrane^{9, 10}. The S protein is then cleaved by the human transmembrane serine protease 2 (TMPRSS2) enzyme^{11, 12}, resulting in membrane fusion between the virus and the human cell¹³. This membrane fusion has been reported to be prevented by the synthesized TMPRSS2 inhibitors nafamostat and camostat (**Figure 1a**), which are approved in Japan for the treatment of acute/chronic pancreatitis^{14, 10}. Nafamostat and camostat have similar basic structures, and both inhibit viral entry into human cells by binding to TMPRSS2.

A crystallographic study revealed that the ester group of nafamostat reacts with the Ser441 side chain in TMPRSS2 to form a covalently bound acyl intermediate¹⁵ (**Figure 1b**). The proposed mechanism of the TMPRSS2 enzymatic reaction is shown in **Figure 1c**¹⁶. Camostat also forms a chemically identical acyl intermediate, as evidenced by the crystal structures of other serine proteases, such as prostasin¹⁷ and enteropeptidase¹⁸. These findings imply that nafamostat and camostat can be classified as so-called covalent drugs that bind to their target proteins through covalent bond formation¹⁶.

Several previous studies have demonstrated the inhibitory activity of nafamostat and camostat against TMPRSS2^{14, 10, 19}. Yamamoto *et al.* measured the half maximum effective concentration (EC₅₀) of nafamostat and camostat using SARS-CoV-2-infected Calu-3 cells. The results showed that the EC₅₀ of nafamostat (1-10 nM) was approximately 1/10 that of camostat¹⁴, indicating that nafamostat can inhibit TMPRSS2 more potently than camostat.

Despite forming chemically identical acyl intermediates, nafamostat and camostat exhibit different TMPRSS2 inhibitory activity. This fact raises the question: how does nafamostat work more effectively than camostat? These two compounds have similar basic structures but differ on one side (**Figure 1a**): nafamostat consists of an amidino group, while camostat comprises ester and amide moieties. This structural difference causes significant difference in their inhibitory effect.

To investigate the differences in the potency of TMPRSS2 inhibitors, we previously calculated the binding affinity of nafamostat, camostat, and five nafamostat derivatives for TMPRSS2²⁰. Notably, our calculations characterized the binding between TMPRSS2 and the compounds as a non-covalent interaction because drug potency is generally discussed in terms of binding affinity to the target protein via the classical treatment. The calculations indicated the nafamostat derivative where the ester bond of nafamostat was replaced with an amide bond (compound 1) as having the highest binding affinity. Based on this result, we expected compound 1 exhibiting the strongest inhibitory activity²⁰; however, *in vitro* assays using SARS-CoV-2-infected cells demonstrated that compound 1 had the weakest inhibitory activity²⁰. Therefore, it was inferred that the antiviral activity of these agents cannot be explained in terms of binding affinity.



Figure 1. (a) Chemical structures of nafamostat, camostat, and compound **1**. (b) Crystal structure of TMPRSS2 forming an acyl intermediate with nafamostat (Protein Data Bank (PDB) ID: 7MEQ²¹). (c) Proposed reaction mechanism of serine protease^{22, 23}. Nafamostat and camostat inhibit TMPRSS2 by covalently binding to Ser441¹⁶. (d) Structures of the reactant, tetrahedral intermediate, and acyl intermediate states optimized by the cluster + PCM model.

The difference in TMPRSS2 inhibitory activity of these compounds may be attributed to the process of their covalent bond formation with TMPRSS2. In other words, the ease of covalent bond formation with TMPRSS2 may contribute to the inhibitory activity. The aforementioned binding affinity factors in the interaction energy between the compound and TMPRSS2 when forming a non-covalent complex²⁰. In contrast, the formation of covalent bonds corresponds to the occurrence of a chemical reaction and strongly depends on the relative energy of the product to the reactant and the activation energy in the chemical reaction²⁴. Therefore, quantitative analysis of the free energy profile during acylation with TMPRSS2 is necessary to examine the covalent bond formation of TMPRSS2 inhibitors.

Classical methods, such as docking simulation and molecular dynamics simulations, are widely used in drug discovery^{25, 26}; nevertheless, these techniques are not suitable for analyzing the process of covalent bond formation²⁷. In contrast, quantum chemical calculations are effective for investigating reaction pathways and are suitable for this task²⁸. However, the quantum mechanical (QM) treatment of the protein environment, which is composed of thousands of atoms, is impractical due to the enormous computational cost^{29, 30}.

To circumvent this problem, this study adopts a combined approach including the molecular cluster model³¹ and the polarizable continuum model $(PCM)^{32}$, and applies it to the analysis of acylation reactions for TMPRSS2 inhibition. In this approach, molecules in the active site are treated quantum mechanically, and the remaining protein environment is approximated as a polarizable continuum³³. This method is hereafter referred to as the cluster + PCM model.

The aim of this study is to elucidate the molecular mechanism underlying the inhibitory activity of TMPRSS2 inhibitors in terms of the ease of covalent bond formation by means of the cluster + PCM model. To this end, the free energies of the reactants, products, and transition

states (TSs) of the three TMPRSS2 inhibitors were calculated along the reaction coordinates, focusing on the differences in the effects of i) nafamostat and camostat and ii) nafamostat and compound **1**. The calculations allowed us to identify the most prominent differences in their inhibitory activities both from a thermodynamic and reaction kinetics aspect.

We first performed geometry optimization³⁴ using the cluster + PCM model on the reactants, tetrahedral intermediates, acyl intermediates, and the two TSs between them. The resulting structures are presented in **Figure 1d**. The detailed structures are shown in **Figure S1** (Supporting Information, SI). Nafamostat and compound **1** did not show significant conformational changes between the five states (reactant, TS1, tetrahedral intermediate, TS2, acyl intermediate), whereas camostat exhibited large orientational changes in the ester and amide moieties. These structural differences probably resulted from the lack of a cationic amidino group on one side of camostat.

The energy profiles corresponding to these structures are displayed in **Figure 2a.** The energies of the acyl intermediates of nafamostat and camostat were 6.63 and 0.65 kcal/mol lower than their reactants, respectively, implying that they both produced stable acyl intermediates. In contrast, compound **1** produced an unstable acyl intermediate that was 16.00 kcal/mol higher in energy than the reactant. These results showed that the stability of the acyl intermediates varies widely among the three compounds.



Figure 2. (a) Free energy profiles of TMPRSS2 inhibitors obtained by the cluster + PCM model. Nafamostat, camostat, and compound **1** are indicated by blue, red, and green, respectively. (b) Free energy profiles of TMPRSS2 inhibitors obtained by the ONIOM method. (c) Boltzmann distribution ratio of acyl intermediates to reactants at 298 K. (d) Schematic illustration of the acylation reaction of TMPRSS2 inhibitors with and without medium change. (e) Rate constant ratio of camostat and compound **1** to nafamostat at 298 K. (f) Coefficient of determination (R^2) between pEC₅₀ and each energy difference (ΔG_1 , ΔG_2 , ΔG_1^{\ddagger} , and ΔG_2^{\ddagger}).

To further examine these results, we used the "our own n-layered integrated molecular orbital and molecular mechanics" (ONIOM) method³⁵ to describe the protein environment of TMPRSS2 in more detail. Here the ONIOM calculations were performed only for the reactant, tetrahedral intermediate, and product, and not for the TS1 and TS2 states. As shown in **Figure 2b**, the ONIOM optimization yielded relative trends in the energy profiles similar to those produced by the cluster + PCM model, although the absolute values relatively differed. These results confirm that the cluster + PCM model adequately represents the protein environment. Therefore, the energy profiles calculated from the cluster + PCM model were used in the subsequent analyses.

The calculated free energies were used to estimate the ratio of the Boltzmann distribution of the acyl intermediate to the reactant (ρ_A/ρ_R) at 298 K, according to the following equation:

$$\frac{\rho_{\rm A}}{\rho_{\rm R}} = \exp\left(-\frac{G_{\rm A} - G_{\rm R}}{RT}\right) \tag{1}$$

where *R* and *T* are the gas constant and temperature, respectively, while G_A and G_R are the free energies for the acyl intermediate and reactant, respectively. The results are summarized in **Figure 2c**. The ratios were calculated to be 7.59×10^4 , 3.04×10 , and 1.65×10^{-12} for nafamostat, camostat, and compound **1**, respectively, indicating that the acyl intermediate is more likely to be formed with nafamostat and camostat, and less likely to be formed with compound **1**. These thermodynamic analyses successfully reproduced a trend in the inhibitory activities of the three compounds which was obtained from *in vitro* studies²⁰.

We now discuss the relationship between the results of the previous *in vitro* assay²⁰ and this energy profile. As stated previously, nafamostat possesses stronger anti-SARS-CoV-2 activity than camostat¹⁴. This experimental result was obtained under the condition of medium

change²⁰; in contrast, camostat showed stronger antiviral activity without medium change²⁰. In other words, the potency of TMPRSS2 inhibitors is strongly dependent on the medium conditions. As illustrated in **Figure 2d**, the no-medium-change condition implies that the leaving groups generated by acylation are not removed during the reaction. Therefore, the concentration of the leaving group in the system gradually increases with the time evolution of the reaction. The higher concentration of the leaving group promotes the reverse reaction of acylation, resulting in a higher concentration of the tetrahedral intermediate. These findings suggest that the tetrahedral intermediate plays a significant role in TMPRSS2 inhibition under the condition of no medium change. As shown in **Figure 2a**, nafamostat was most stable in the acyl intermediate state (6.63 kcal/mol more stable than the reactant), while camostat was most stable in the tetrahedral intermediate state (2.84 kcal/mol more stable than the reactant). In addition, camostat forms a more stable tetrahedral intermediate than nafamostat under the no-medium-change condition is attributed to its more stable tetrahedral intermediate state.

Next, the TMPRSS2 inhibitory activity of nafamostat and camostat was investigated in terms of reaction kinetics. As shown in **Figure 2a**, the activation energies of nafamostat in TS1 and TS2 were 7.74 and 0.96 kcal/mol, respectively, while those of camostat were 11.14 and 2.81 kcal/mol, respectively. Thus, the activation energies of nafamostat were found to be 3.40 and 1.85 kcal/mol lower than those of camostat in TS1 and TS2, respectively. These results indicate that nafamostat is more reactive than camostat.

To gain further insight into the reactivities of these compounds, we estimated the ratio of the rate constants of camostat and compound **1** to nafamostat at 298 K. Assuming that the transmission factor in the Eyring equation for all three compounds is the same^{36, 37}, the relative reaction rate k_i/k_{Naf} (for *i* = camostat or compound **1**) is determined as follows:

$$\frac{k_i}{k_{\text{Naf}}} = \exp\left(-\frac{\Delta G_i^{\ddagger} - \Delta G_{\text{Naf}}^{\ddagger}}{RT}\right)$$
(2)

where ΔG_i^{\ddagger} and $\Delta G_{Naf}^{\ddagger}$ are the activation energies of molecule *i* (for *i* = camostat or compound **1**) and nafamostat, respectively. The results of the relative rates are summarized in **Figure 2e**. In TS1, they were calculated to be 3.07×10^{-3} and 2.62×10^{-3} for camostat and compound **1**, respectively. These results indicate that nafamostat is 3.25×10^2 and 3.82×10^2 times more reactive than camostat and compound **1**, respectively. In TS2, the calculated relative rates were 4.22×10^{-2} and 5.22×10^{-5} for camostat and compound **1**, respectively, indicating that the reactivity of nafamostat is 2.37×10 and 1.92×10^4 times higher than those of camostat and compound **1**, respectively. Thus, the kinetic analysis of Eq. (2) demonstrated that nafamostat is more likely to produce an acyl intermediate than camostat and compound **1**.

We next attempted to compare the results of the energy profile analysis with the results of the *in vitro* assay²⁰. To identify the most significant contribution to the anti-SARS-CoV-2 effect, we estimated the correlations of ΔG_1 , ΔG_2 , ΔG_1^{\ddagger} , and ΔG_2^{\ddagger} to pEC₅₀ ($-\log_{10}[EC_{50}]$)²⁰. Here, ΔG_1 and ΔG_2 represent the free energy differences from the reactant to the tetrahedral and acyl intermediate states, respectively, and ΔG_1^{\ddagger} and ΔG_2^{\ddagger} are the activation energies of TS1 and TS2, respectively. The definition of each energy difference is shown in **Figure 2a**. **Figure 2f** summarizes the coefficient of determination (R^2) between pEC₅₀ and each energy difference (see **Figure S2** for details). The R^2 values were calculated to be 0.906, 0999, 0.497, and 0.993 for ΔG_1 , ΔG_2 , ΔG_1^{\ddagger} , and ΔG_2^{\ddagger} , respectively. Thus, all three energy differences except ΔG_1^{\ddagger} were highly correlated with pEC₅₀; the value for ΔG_2 showed the largest correlation, suggesting that the stability of the acyl intermediate state majorly contributes to the TMPRSS2 inhibitory activity.

We further investigated the cause of the stability in the acyl intermediates of nafamostat. As shown in **Figure S1**, both nafamostat and compound **1** produce acyl intermediates with the same structure. However, the formation of the acyl intermediate results in the production of different types of leaving groups for nafamostat and compound **1**. In nafamostat, the leaving group consists of a naphthoxide moiety, while in compound **1**, it consists of a naphthylamide moiety (**Figure 3a**). To analyze the effect of these leaving groups, we calculated the interaction energies between the leaving groups and the acyl intermediates as follows:

$$G_{\rm Int} = G_{\rm All} - \left(G_i + G_{\rm LG}\right) \tag{3}$$

where G_i and G_{LG} denote the free energies of the acyl intermediate (Ser441) and the leaving group, respectively, and G_{All} is the free energy of their complex. The interaction energies for nafamostat and compound **1** were calculated to be -4.32 and -0.52 kcal/mol, respectively, confirming that the leaving group of nafamostat produces a more stable structure. We further extended the interaction analysis of the leaving group to the amino acids in the active site (i.e., Asp345, His296, Asp435, Glu299, and Gly391). Here, the free energy of amino acid *i* (for *i* = Asp345, His296, Asp435, Glu299, and Gly391) was employed for G_i in Eq. (3). The structures of these amino acids are shown in **Figure 3a**. The results are summarized in **Figure 3b**. The strongest interaction was obtained from Glu299, with interaction energies of -37.89 and -30.78 kcal/mol for nafamostat and compound **1**, respectively. The total interaction energies were calculated to be -2.53 and 3.38 kcal/mol for nafamostat and compound **1**, respectively. The difference between their total interaction energies was 5.91 kcal/mol, which accounts for as much as a quarter of the energy difference between the acyl intermediates of nafamostat and compound **1** in the energy profiles (22.63 kcal/mol). Thus, the stability of the acyl intermediate of nafamostat is attributed to the strong interaction of its leaving group with the acyl intermediate and surrounding protein. This result is more clearly understood by the electrostatic potential (ESP) maps of the acyl intermediate state shown in **Figure 3c**. The naphthoxide moiety of nafamostat generates a larger negative ESP than the naphthylamide moiety of compound **1**. This fact indicates that the leaving group of nafamostat attracts the acyl intermediate more strongly than that of compound **1**, stemming from the presence of the positive charge derived from the guanidino group of the acyl intermediate. These analyses successfully demonstrate that the more negative ESP generated by the nafamostat leaving group directly contributes to the stability of its acyl intermediate. The Mulliken population analyses also support these results (see **Figure S3** for details).



Figure 3. (a) Structures of the amino acids in TMPRSS2 used to calculate the interaction energies with the leaving group. (b) Interaction energies of the leaving groups with the acyl intermediates and amino acids for nafamostat and compound **1**. (c) Electrostatic potential (ESP) map generated by the leaving groups and acyl intermediates of nafamostat and compound **1**.

The origin of the stability of the tetrahedral intermediate was also investigated. The formation of the tetrahedral intermediate requires the oxygen atom of the Ser441 side chain to attack the carbonyl carbon of the TMPRSS2 inhibitor. To examine this effect, the orbital interaction between the TMPRSS2 inhibitor and Ser441 were estimated. Here, we focused on the molecular orbitals (MOs) distributed on Ser441 and the TMPRSS2 inhibitors before and after the orbital interaction. The shapes of reactive orbitals are displayed in Figure 4a. The orbitals after interaction are shown in **Figure S4**. Notably, only the catalytic triad (Asp435, His296, Ser441) and the TMPRSS2 inhibitor were calculated as QM atoms, while the other amino acids were incorporated as point charges. Figure 4b illustrates the orbital interactions between the occupied orbitals distributed on Ser441 (highest occupied MO -6 (HOMO-6), HOMO-4, and HOMO-5 for nafamostat, camostat, and compound 1, respectively) and the lowest unoccupied MOs (LUMOs) of the TMPRSS2 inhibitors. The magnitudes of the orbital interactions (orbital energy gaps) were calculated to be 4.98, 4.40 and 4.15 eV for camostat, nafamostat, and compound 1, respectively. As already shown in **Figure 2**, the analysis of the energy profiles revealed that the stability of the tetrahedral intermediates was in the order of camostat, nafamostat, and compound 1. Thus, the magnitude of the orbital interactions was in good agreement with the trend of the energy profiles. Further MO analysis revealed that the ester moiety of camostat and the OH group of Ser441 faced each other, resulting in a larger orbital overlap between them (Figure 4a). By conjointly considering the above, the stability of the tetrahedral intermediate of camostat was inferred to result from the larger orbital interaction between camostat and Ser441.



Figure 4. (a) Shape of molecular orbitals (MOs) involved in the formation of tetrahedral intermediates. (b) Orbital interactions during the formation of tetrahedral intermediates.

In this study, the TMPRSS2 inhibitory activity of nafamostat, camostat, and compound **1** was investigated using the cluster + PCM model and the ONIOM method. The calculations of the energy profiles including the reactants, tetrahedral intermediates, acyl intermediates, and their two TS states clearly indicated that nafamostat proceeds the acylation reaction more efficiently than camostat. The calculations also showed that despite its structural similarity to nafamostat, compound **1** is not a suitable TMPRSS2 inhibitor. Since these results could not be derived from conventional binding affinity calculations, this study highlights for the first time the significance of energy profile analysis in the investigation of TMPRSS2 inhibitory activity.

The present study demonstrates the potent TMPRSS2 inhibitory activity of nafamostat both from a thermodynamic and kinetic point of view, which is attributed to the formation of a stable acyl intermediate and its low activation energy.

The cluster + PCM model employed in this study offers simplicity and broad prospects for combination with other methods. In our future work, this approach will be used to elucidate the molecular mechanisms of other covalent drugs with the aim of developing new effective antiviral agents.

ASSOCIATED CONTENT

The following files are available free of charge.

Details of the computational procedures using the cluster + PCM model and the ONIOM method; optimized structures of nafamostat, camostat, and compound **1** by the cluster + PCM model; scatter plots between the experimental pEC₅₀ and the free energy difference (ΔG_1 , ΔG_2 , ΔG_1^{\ddagger} , and ΔG_2^{\ddagger}); Mulliken charge distribution of the leaving groups and surrounding amino acids in acyl intermediate states; shape of molecular orbitals of tetrahedral intermediates involved in the acylation; atomic coordinates of the optimized structures of nafamostat, camostat, and compound **1** in five states (reactant, TS1, tetrahedral intermediate, TS2, and acyl intermediate) (PDF).

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Notes

The authors declare no competing financial interests.

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