Design of galardine analogs as putative psudolysin inhibitors based on ab initio fragment molecular orbital calculations

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Abstract

Pseudolysin (PLN) is a metalloproteinase secreted from bacteria that degrades extracellular proteins to produce bacterial nutrition. It is thus expected that inhibitors against PLN can suppress the growth of bacteria and their pandemic spread. In addition, since these inhibitors do not attack bacteria directly, there is a reduced risk for producing drug-resistant bacteria.. Since human matrix-metalloproteinases (MMPs) have large structural similar in the active sites with PLN, there is a possibility that the inhibitors for PLN also inhibit MMP activity, resulting in a loss of necessary nutrients to be produced by MMPs. Therefore, agents inhibiting the activity of only PLN and not MMP are required. In the present study, we employed a hydroxamate compound galardin, which has a significant inhibition effect against PLN and MMP, and investigated its specific interactions with PLN/MMP at atomic and electronic levels, by use of *ab initio* molecular simulations. Based on the results, we proposed several derivatives of galardin and elucidated which derivatives that can bind more strongly to PLN than MMPs and be potent antimicrobial agents capable of inhibiting the PLN activity.

Keywords

Zinc metalloproteinases; Bacterial virulence; Molecular simulation; Fragment molecular orbital; Protein-ligand interactions; Drug design

1. Introduction

Antimicrobial agents inhibiting the growth of bacteria have been used widely to treat infectious diseases. However, the emergence of drug-resistant strains of bacteria and their spread pose a major threat to humans. In fact, the World Health Organization (WHO) has disseminated the message "Antimicrobial resistance: no action today, so cure tomorrow" in April 2011 [1, 2]. Therefore, it is urgently required to develop novel potent drugs against bacterial infections with a low risk of drug-resistance.

Bacteria secrete an enzyme pseudolysin (PLN) to degrade extracellular proteins and peptides. These degraded substances are used as a nutrient necessary for bacterial growth [3]. PLN contains Zn and Ca ions. The Ca ion contributes to stabilizing the PLN structure, while the Zn ion is important for the enzyme activity. Zn ion is located around the active sites of PLN, which recognize the proteins and peptides to be degraded. The S1' pocket composed of Leu132 and Val137 residues and the S2' pocket (Asn112, Phe129, Ile186, Leu197 and Arg198) recognize hydrophobic amino acids, while the S1 pocket (Tyr114, Trp115 and Tyr155) recognizes hydrophilic amino acids. Among these pockets, the S1' pocket is the most important for the activity of PLN [3, 4]. Therefore, if the function of PLN can be effectively inhibited by binding an agent to the S1' pocket, it is expected that bacterial growth can be suppressed. With this purpose, the development of PLN inhibitors may be a novel strategy for the development of new antimicrobial agents [3, 4]. Since these agents do not directly attack bacteria, there is a reduced risk for producing drug-resistant bacteria.

On the other hand, cells in a living organism employ biologically inherent matrix metalloproteinases (MMPs) to degrade exocellular proteins, and the produced peptides are used as a nutrient for cells [5]. MMPs are proteolytic enzymes originally present in living organisms that plays an important role in the degradation of extracellular matrix. Currently, 23 different MMPs have been identified in mammals [6]. Among the MMPs, MMP-9 plays an essential role in the differentiation and proliferation of tissue stem cells and the regeneration of peripheral tissues [7]. Normally, the activity of MMP-9 is balanced by the tissue inhibitor of metalloproteinases (TIMPs). However, an excessive expression and activity of MMP-9 cause excessive degradation of extracellular proteins and excessive destruction of cellular tissues, inducing invasion and metastasis of cancer cells. MMP-9 contains two Zn ions, which play a significant role for the enzyme activity of MMP-9, and a Ca ion that stabilizes the structure of MMP-9. In MMP-9, the ligand-binding pocket S1', composed by the Asp185-Leu188 and Pro246-Tyr248 amino-acid residues, has a great influence on the MMP-9 activity for the peptide hydrolysis [7].

Both PLN and MMP-9 have Zn ions in their active sites, and the structures of

their ligand binding sites are similar to each other [3], as shown in Figure S1 of the supplementary information. Therefore, there is a possibility that the inhibitors for PLN also inhibit the enzymatic activity of MMP-9, resulting in a loss of necessary compounds produced by MMP-9. In order to prevent such side effects, it is necessary to develop inhibitors selectively acting on PLN. However, since the structures around the active sites of MMP-9 and PLN are similar to each other, it is difficult to develop such a selective inhibitor. To overcome this issue, we attempted to elucidate the specific binding properties between PLN/MMP-9 and the existing ligands (ARP101 and LM2) at atomic and electronic levels, using *ab initio* molecular simulations [8], in which protein-ligand docking, molecular mechanics (MM) optimization and *ab initio* fragment molecular orbital (FMO) methods were used.

In a recent experimental study [9], the binding strengths of PLN and MM-9 in addition to other metalloproteinases with two hydroxamate compounds galardin and **1b** were determined. The chemical structures of these compounds are shown in Figure S2 of the supplementary information. By using molecular docking simulations, the specific interactions between the metalloproteases and these compounds were investigated to find that the S1' pocket of PLN is too small to accommodate the diphenyl ether moiety of **1b**, while the 4-methylpentanoyl moiety of galardin enters the pocket. This study clearly indicates that the size and shape of the ligand structural moiety entering the S1' pocket is an important determinant for selective interactions between the metalloproteases and these compounds.

In the present study, we furthermore investigated the specific interactions between PLN/MMP-9 and galardin, which was found to bind strongly to the S1' pocket of PLN in the previous experiment [9]. Based on the results simulated, we proposed several derivatives of galardin and elucidated *in silico* which derivatives that can bind more strongly to PLN than to MMP-9, and be putative potent antimicrobial agent capable of inhibiting the PLN activity. The present results produce useful information for developing novel antibacterial agents targeting PLN.

2. Details of *ab initio* molecular simulations

2.1. Structure optimizations of MMP-9+ligand and PLN+ligand complexes

To obtain the initial 3d-structures of MMP-9 and PLN, we first extracted the structures of MMP-9 and PLN from the structures of their complexes with ligand, which are registered in the protein data band (PDB). The PDB IDs for these structures are 5CUH [10] for MMP-9 and 1U4G [11] for PLN. Using the ligand-docking option of the international computer management (ICM) software, galardin was docked to a similar

position as the ligand in the PDB structure to obtain the initial structures of the MMP-9+galardin and the PLN+galardin complexes.

The chemical structure of galardin is shown in Figure S2, and its inhibitory effect (Ki values) against MMP-9 and PLN obtained by the experiment [9] are 0.051 and 20 nM, respectively, indicating that galardin is more effective inhibitor of MMP-9 than of PLN. It is expected that the two oxygen atoms of the hydroxamic group of galardin contribute significantly to the binding to the Zn ions of metalloproteinases MMP-9 and PLN. The structure of galardin was optimized in vacuum by the B3LYP/6-31G(d,p) calculation of Gaussian09 (G09) [12], and the charge distribution of the optimized structure was analyzed by the HF/6-31G(d) method of G09. Based on this charge distribution, atomic charge parameters of the force fields employed in the MM and molecular dynamics (MD) simulations were constructed.

The initial structures of the complexes were fully optimized in water using the classical MM and MD simulation program AMBER12 [13]. In order to properly consider the solvation effect on the complex, we added water molecules with a layer of 8 Å around the complex. The total number of water molecules considered is around 1200 for the MMP-9 complex and 1700 for the PLN complex. In the MM optimizations, AMBER99SB-ILDN force field [14], TIP3P model [15] and the generalized AMBER force field (GAFF) [16] were used for protein, water molecules and ligand, respectively. The threshold value of energy gradient for convergence in the MM optimization was set as 0.0001 kcal/mol/Å. The N- and C-termini of MMP-9 and PLN were terminated by acetyl and methylamine groups, respectively. In the previous molecular simulations [17] of the metalloproteinase thermolysin, whose structure is very similar to the PLN structure, a proton was added to the carbonyl group of Glu141 side-chain, in order to neutralize the change of Glu141 and reproduce the structure of thermolysin obtained by the experiment. In the present study, we added a proton in the same way.

2.2. FMO calculations for analyzing electronic states of the complexes

To elucidate the specific interactions between MMP-9/PLN and galardin, the electronic properties for the solvated structures of the complexes were investigated by the *ab initio* FMO method [18]. We employed the water molecules existing within 8 Å from the galardin explicitly, in order to consider the effect of water molecules on the interactions between MMP-9/PLN and galardin.

In FMO calculations, the target molecule is divided into units, each of which is called “fragment”, and the electronic properties of the target molecule are estimated from the electronic properties of the monomers and the dimers of the fragments. The specific

interactions between the fragments can be investigated from the interaction energies obtained by the FMO calculation. In the present FMO calculations, ligand, each residue of the protein and each water molecule were assigned as a fragment, because this fragmentation enables us to evaluate the interaction energies between the ligand and each residue of the protein. It is noted that Zn ion and some residues coordinated with Zn are considered in the same fragment, because they strongly interact with each other. In fact, for the MMP-9+ligand complexes, Zn1 is included in the same fragment of Hid226, Hid230 and Hid236, while Zn2 is included in the same fragment as Hid175, Asp177, Hid190 and Hie203. On the other hand, PLN has only one Zn ion, which is included in the same fragment as Hid140, Hid144 and Glu164.

The FMO calculation program ABINIT-MP Ver.6.0 [19] was used in the present study. The *ab initio* MP2[20, 21]/6-31G method of FMO was employed to accurately investigate the π–π stacking, NH–π and CH–π interactions between the galardin and the residues of MMP-9/PLN. In addition, to elucidate which residues of MMP-9/PLN contribute to the binding to galardin, we investigated the inter fragment interaction energies (IFIE) [22] obtained by the FMO calculation.

Furthermore, to determine which ligand can bind more strongly to the target proteins (MMP-9 and PLN), we evaluated the binding energy (BE) between protein and ligand from the total energies (TEs) of the component structures using the following equation.

BE =TE(Protein+ligand+water) −TE(Protein+water) −TE(Ligand+water)+TE(Water)

In the above calculations, we employed the structure of the solvated complex optimized by the classical MM method. From this structure, we extracted the structures of protein+water, ligand+water and only waters, and the TEs of these structures as well as the solvated complex were evaluated by the *ab initio* FMO calculations.

2.3. MD simulations for confirming the stability of the complexes

To confirm that our proposed galardin derivative keeps its binding to PLN even at room temperature, we finally carried out MD simulations in explicit waters, using the MM and MD simulation program GROMACS Ver. 4.5.3 [23]. We considered a cubic water box, whose size is 2 times as larger as the longest diameter of the complex and executed the MD simulation by setting the periodic boundary condition in the XYZ directions with the water box as a unit. The same force fields as the MM optimizations were employed in the MD simulations.

At first, the stable structures of the complexes in water were determined by the

MM optimization of GROMACS. Subsequently, structural equilibrium calculation was executed by 1 ns MD simulations under a constant temperature and pressure condition (300 K, 1 atm), in order to relax the position and the density of the solvating water molecules. After optimizing the size of the water box, a 30 ns MD simulation was conducted under constant temperature and volume conditions at 300 K. From the trajectories obtained by the 30 ns MD simulation, we analyzed the root mean square deviation (RMSD) from the initial structure of the complex to elucidate the changes in structure during the MD simulation.

3. Results and discussion

3.1. Specific interactions between MMP-9/PLN and galardin

To check the adequacy of the present molecular simulations, we first investigated the BEs between galardin and MMP-9 or PLN and compared the results with the Ki values obtained by the previous experiment [9]. The BEs are evaluated to be −261.0 (MMP-9) and −101.6 (PLN) kcal/mol, respectively. This result can explain the trend of the Ki values (0.051 nM for MMP-9 and 20 nM for PLN), indicating that galardin is more effective to MMP-9 than PLN. Therefore, it was confirmed that the present molecular simulation can explain the trend of binding strength of galardin to MMP-9 and PLN.

To elucidate the reason for the significant difference in BEs of galardin for MMP-9 and PLN, we investigated the interaction energies between galardin and the amino acid residues of these metalloproteases. As shown in Figure 1a, in the MMP-9+galardin complex, galardin interacts very strongly with the fragment, which contains Zn1, Hid226, Hid230 and Hid236. The interaction energy is more than 10 times larger than those for the other residues of MMP-9. Therefore, it is revealed that galardin binds specifically to the Zn1 group of MMP-9. In addition, Leu187, Met247, Tyr248 and the other Zn2 group contribute to the binding to galardin, as shown in Figure 1b.

On the other hand, in the PLN+galardin complex, the Zn group including Hid140, Hid144 and Glu164 of PLN interacts less strongly with galardin as shown in Figure 2a. The interaction energy is about the fifth of that between galardin and the Zn1 group of MMP-9. It is also elucidated from Figure 2b that galardin interacts attractively with Asn112, Tyr114 and Arg198 and interacts repulsively with Asp168. From the comparison of the IFIEs shown in Figures 1 and 2, it is elucidated that the main reason for the large BE of galardin for MMP-9 is the strong attractive interaction between galardin and the Zn1 group of MMP-9. In other words, the ligand binding pocket around the Zn1 ion of MMP-9 is more suitable for the strong binding of galardin to the pocket than that of PLN.

Furthermore, we compared the interacting structures between galardin and the

residues of these metalloproteases to reveal the origin for the difference in the above mentioned interaction energies. As shown in Figure 3a, the two oxygen atoms of the hydroxamic group of galardin strongly coordinate with Zn1 at 1.85 and 2.56 Å distance, resulting in the very large attractive interaction energy between galardin and Zn1 in the MMP-9+galardin complex. Figure 3b indicates that the other Zn ion (Zn2) included in MMP-9 exists far away from galardin, however, the oxygen atom of the backbone of Hid190, which is coordinated with Zn2, forms a hydrogen bond with the hydrogen atom of galardin. As a result, the interaction energy between galardin and the Zn2 group is large as shown in Figure 1b. In addition, Figure 3c indicates that the oxygen atom of galardin electrostatically interacts with the hydrogen atoms of the Leu187 backbone, and that the oxygen atom of the Met247-Tyr248 backbone forms a hydrogen bond with the hydrogen atom of galardin. The other part of Tyr248 also forms a hydrogen bond with the oxygen atom of galardin at 1.87 Å distance. Therefore, it is elucidated from Figure 3 that galardin can insert into the S1' ligand-binding pocket of MMP-9 and bind strongly with Zn1 ion and some residues existing around the S1' pocket, resulting in the high binding affinity of galardin to MMP-9.

In contrast, as shown in Figure 4a, the interacting structures between PLN and galardin are significantly different from those for MMP-9. Only one oxygen atom of the hydroxamic group of galardin is coordinated with the Zn ion of PLN, resulting in smaller interaction energy between galardin and the Zn group of PLN compared with that for the MMP-9+galardin complex. Figure 4a also elucidates that a negatively charged Glu164 residue exists near the ligand-binding pocket of PLN, and Zn ion is tremendously attracted to the COO^- group of the Glu164 sidechain. As a result, Zn progresses more deeply into the pocket, so that it becomes difficult for the two oxygen atoms of galardin to coordinate with the Zn ion of PLN.

Figure 2b indicates that the other residues of PLN also contribute to the binding of galardin. The interacting structures of galardin and these residues are shown in Figure 4b. The oxygen atom of the Asn112 backbone and the hydrogen atom of galardin form a strong hydrogen bond, while the hydrogen atom of the Asn112 sidechain forms a hydrogen bond with the oxygen atom of galardin. These hydrogen bonds create large attractive interaction energy between galardin and Asn112. In addition, there are some hydrogen bonds between galardin and Tyr114 and Arg198. As mentioned above, the future of IFIEs between galardin and the PLN residues shown in Figure 2b can be explained qualitatively by the interacting structures shown in Figure 4.

As mentioned above, the interaction between the hydroxamic group of galardin and Zn ion included in the metalloproteases was found to significantly affect the binding affinity between galardin and the metalloproteases. We therefore compared the interactions in the MMP-9+galardin and the PLN+galardin complexes in Figure 5. As shown in Figure 5a, Zn1 of MMP-9 exists between the two oxygen atoms of the hydroxamic group of galardin and coordinates with both oxygen atoms, resulting in very strong attractive interactions between galardin and the Zn1 group. On the other hand, Figure 5b indicates that Zn of PLN exists near one oxygen atom of galardin and forms only one coordination bond with the oxygen atom of galardin. The comparison of the structures shown in Figure 5 elucidates that the difference in the coordination bonds between Zn and the oxygen atoms causes the significant difference in the binding affinity between galardin and MMP-9 or PLN. Accordingly, it is expected that the binding between PLN and galardin can be enhanced, if the position of Zn in PLN is shifted into the place between the two oxygen atoms of the hydroxamic group of galardin, or if the two oxygen atoms are shifted nearer to the Zn of PLN.

3.2. Proposal for potent inhibitors against PLN by manual operation

In order to propose potent PLN inhibitors, we considered some galardin derivatives and investigated their binding properties with PLN, using the *ab initio* FMO calculations. We first proposed the three derivatives G1, G2 and G3, whose chemical structures are shown in Figure 6. To decrease the size of galardin, the butyl group of galardin was replaced by a hydrogen atom to obtain the G1 derivative (Figure 6a), while the ring group and the carbon atom were replaced by a hydrogen atom to make the G2 derivative (Figure 6b). In the G3 derivative (Figure 6c), both groups were replaced by hydrogen atoms to make a small compound. These derivatives are expected to move more easily in the ligand-binding pocket of PLN than galardin and coordinate strongly with the Zn ion of PLN.

The structures of PLN with these derivatives were fully optimized in water by the MM method, and the BEs between PLN and the derivatives were investigated by the *ab initio* FMO calculations. As listed in Table 1, our proposed galardin derivatives have larger BEs than galardin, indicating their stronger binding to PLN. In particular, the BE of the derivative G3 is 27 kcal/mol larger than that of galardin. To elucidate the reason for this large BE, we moreover analyzed the interactions between G3 and the PLN residues. As shown in Figures S3, S4 and S5 of the supplementary information, all derivatives have strong attractive interactions with the Zn group of PLN. The interaction energy between the Zn group and each of the derivatives was evaluated to be −74.3 (G1),

−221.8 (G2) and −232.3 kcal/mol (G3), respectively. The small sized G3 has the largest interaction energy, since it can move more freely in the ligand-binding pocket of PLN and coordinate more strongly with the Zn ion. On the other hand, the interactions between these derivatives and the other PLN residues are not affected significantly by the replacement introduced in galardin. In fact, G2 and G3 interact strongly with Asn112, Tyr114 and Arg198, in the same way as galardin.

To clarify the reason for this change in the interaction energies induced by the change of the derivatives, we compared the interacting structures between PLN and these derivatives. In particular, we focused on the interactions between Zn ion and the derivatives. As shown in Figure S6a, only one oxygen atom of the hydroxamic group of G1 is coordinated with Zn ion, resulting in the small (−74.3 kcal/mol) interaction energy. This interaction is similar to that for the PLN+galardin complex. On the other hand, in the PLN+G2 (Figure S7a) and PLN+G3 (Figure S8a) complexes, two oxygen atoms of the hydroxamic group of G2 or G3 are strongly coordinated with Zn ion. Due to these coordinations, G2 and G3 interact strongly with the Zn group of PLN.

In addition, we analyzed the interactions between the derivative and the other PLN residues. As shown in Figures S6b, S7b and S8b, each of the derivatives forms hydrogen bonds with the hydrogen atom of Asn112 sidechain and the oxygen atom of Tyr114 backbone. The hydrogen atom of Arg198 sidechain also forms a hydrogen bond with the derivative (Figures S6c, S7c and S8c). It is noted that galardin and G1 form two hydrogen bonds with Asn112 as indicated in Figure 4b,while G2 and G3 form only one hydrogen bond with Asn112 (Figures S7b and S8b). As a result, G2 and G3 can move more freely in the ligand-binding pocket of PLN and are expected to coordinate more strongly with the Zn ion existing in the pocket.

In order to propose novel potent inhibitors against PLN based on G3, which has the largest BE with PLN, we furthermore investigated in detail the interactions between G3 and the PLN residues and Zn ion around the ligand-binding pockets. As listed in Table 2, among the Tyr114, Trp115 and Tyr155 residues existing near the S1 pocket, only Tyr114 has a strong attractive interaction with G3. In contrast, the Leu132 and Val137 residues existing at the S1’ pocket have no contribution to the G3 binding. As for the PLN residues (Asn112, Phe129, Ile186, Leu197 and Arg198) at the S2’ pocket, only Asn112 and Arg198 interact strongly with G3. In the previous experiments [3, 4], the S1’ pocket of PLN was considered to be important for the ligand binding. We therefore attempted to propose novel derivatives, which can interact with the PLN residues existing at the S1’ pocket as well as the S1 and S2’ pockets. To produce hydrogen bonds with the hydrogen atoms of the residues around the S1’ pocket of PLN, we introduced a hydroxy group or a

carboxy group into G3. In addition, a methyl group was introduced into some G3 sites, in order to enhance hydrophobic interactions with the residues of the S1' pocket. The chemical structures of our proposed compounds based on G3 are shown in Figure 6, in which the symbols "a" and "b" in the last of the name indicate the positions of the replaced parts of G3.

The evaluated BEs between PLN and our proposed compounds are listed in Table 1. Among the six proposed compounds, only G5b and G6b have larger BEs than the pristine G3, while the other compounds have rather small BEs compared with G3. Therefore, it is expected that G3, G5b and G6b can be potent inhibitors of PLN. In particular, G6b has significantly larger BE than G3 and the other derivatives, so that G6b is considered to be the most potent for inhibiting the PLN activity. As for the position of the replacement on G3, the compounds with the replacement of the "b" site prefer to have larger BEs than those with the "a" replacement.

To reveal the reason for the difference in these BEs, we investigated the interaction energies between the compounds and each of the PLN residues and Zn ion. As indicated in Figures S9~S14, all proposed compounds interact most strongly with the Zn group including Hid140, Hid144 and Glu164 of PLN, although the interaction energies significantly depend on the compounds. We analyzed the correlation between the BEs and the interaction energies for our proposed derivatives. As indicated in Figure S15, the correlation coefficient R^2 is 0.67, indicating that these values are well correlated to each other. Therefore, it can be concluded that the compound with strong interaction with the Zn group of PLN has larger binding affinity to PLN.

G6b has the largest BE and interacts most strongly with the Zn group of PLN; its interaction energy is −240 kcal/mol. To make clear the reason for this interaction, the interacting structure between G6b and PLN was analyzed in detail. As shown in Figure 7a, the two oxygen atoms of the hydroxamic group of G6b strongly coordinate with Zn ion at the distance of 2.14 and 2.18 Å, respectively. Additionally, the methyl group introduced into G6b causes hydrophobic interactions with the methyl groups of Leu132 and Val137 at the S1' pocket, as indicated in Figure 7b. Consequently, by introducing a methyl group at the "b" site of G3, we can obtain a potent inhibitor against PLN, that interacts with the residues at the S1' pocket as well as the S1 and S2' pockets. We also confirmed that G5b has a similar binding property as G6b, as shown in Figure S16.

As for the dependence of binding properties between PLN and the compounds on the sites of replacement, the BEs of G5b and G6b are larger than those of G5a and G6a, indicating that the compounds with the b-site replacement prefer to bind more strongly to PLN. To reveal the reason for this dependence, we compared the IFIEs

between PLN residue and the compound for the G4a and G4b, G5a and G5b, and G6a and G6b. As shown in Figure S17, the interaction between the Zn group of PLN and the compound is significantly larger for the compound with the b-site replacement than that with the a-site one. The interactions with Asn112 and Tyr114 are also larger for the compound with the b-site replacement. The replaced group at the b-site of G3 is expected to contribute more preferably to the interactions with the PLN residues and Zn ion.

3.3. Proposal for potent inhibitors against PLN by automatic and global operation

By using the above-mentioned manual operation, we proposed nine derivatives based on galardin shown in Figure 6. Among them, G6b was found to have the largest BE to PLN as listed in Table 1. Since the BE of G6b is significantly larger than that of galardin, G6b is expected to be a potent PLN inhibitor. To search for novel compounds more globally, we utilized SwissBioisostere (database of molecular replacements for ligand design) [24], SwissADME (website for predicting ADME parameters, pharmacokinetic properties, drug like nature and medicinal chemistry friendliness)[25] and PreADMET (web-based application for predicting ADME data and building drug-like library using in silico method) [26]. Among the compounds produced by these methods, we selected some candidate compounds and investigated their binding properties with PLN to elucidate which compound can bind more strongly to PLN.

At first, a series of galardin derivatives were designed by replacing the butyl and the ring groups of galardin shown in Figure 8, using molecular replacements acquired from the SwissBioisostere database [24]. This database is a collection of information on 4.5 million molecular sub-structural replacements and their information in biochemical assays created through the detection of matching molecular pairs and by the process of mining bioactivity data in the ChEMBL database. In total, 2140 derivatives were created by use of the SwissBioisostere database. Among them, we selected the 42 derivatives with high activity and a score larger than 0.85.

Next, using SwissADME [25], the chemical properties of the selected derivatives were checked as listed in Table 3. These derivatives were screened by Lipinski's rules of five [27]; and the Veber rules stating: molecular weight (MW) < 500 Da, number of rotatable bonds (RB) $\leq$ 12, number of H-bond acceptors (HBA) < 10, number of H-bond donors (HBD) < 5, the octanol-water partition coefficient (LogP) < 5,and a polar surface area (PSA) $\leq$ 140 $Å^2$. As listed in Table 3, the red marked properties do not meet the rules. The 14 derivatives meeting the rules were selected as the candidate ligands for PLN.

In addition, we checked if these derivatives meet the conditions of the absorption, distribution, metabolism, excretion and toxicity properties (ADMET rules [26]) or not,

using PreADMET software [26]. PreADMET predicts the values of physically relevant descriptors and pharmaceutically important properties of ligands that can be compared with the recommended values of ideal drugs. In the present study, blood-brain barrier (BBB) penetration, heterogeneous human epithelial colorectal adenocarcinoma cell lines (Caco2), human intestinal absorption (HIA), plasma protein binding (PPB), toxicity to mouse and rat, and hEFG inhibition risk (hEFG) were considered. As listed in Table 4, it was confirmed that all 14 derivatives fulfill the conditions as ideal drugs, although their BBB values are less than 0.1, indicating a low absorption of these derivatives. Among these 14 derivatives, five derivatives with a positive toxicity for carcino-mouse or carcino-rat were eliminated, and the binding properties of the remaining nine derivatives to PLN were investigated by *ab initio* FMO calculations. The chemical structures of the derivatives are shown in Figure 9.

The BEs between PLN and the created derivatives were evaluated in the same manner as for the PLN+galardin complex. The results are listed in Table 5, indicating that six derivatives have larger BE than galardin and may be potent inhibitors of PLN. However, in the above mentioned automatic progress, we cannot find a novel derivative with a BE larger than G6b, which has the largest BE among the derivatives produced by our manual operation.

To elucidate the specific interactions between PLN and these derivatives, we analyzed the interaction energies between each PLN residue and each of the derivatives. As shown in Figures S18-S26, all derivatives interact most strongly with the Zn group of PLN. The interaction energy varies widely in the range from −68 to −270 kcal/mol.

In addition, the interacting structures between PLN and the derivatives were investigated to elucidate the reason for the smaller BEs of the derivatives compared with that of G6b. As shown in Figures S27-S35, the derivatives (N4, N6, N32, N39, N40) with large BE have two oxygen atoms coordinated with Zn ion of PLN. In particular, N32 has two coordination bonds with Zn at 2.03 and 2.07 Å distance to interact most strongly with the Zn group of PLN, as shown in Figure S32a. N32 additionally forms hydrogen bonds with Asn112, Tyr114 and Arg198, as shown in Figures S32b and S32d. Therefore, among the nine galardin derivatives created automatically, N32 has the largest BE, although its BE is smaller than that for G6b created manually by the present study.

3.4. MD simulations for PLN+G6b complex

Finally, we conducted a 30 ns MD simulation in water for the PLN+G6b complex, in order to confirm that G6b can keep its binding to the ligand-binding site of PLN even at 300 K. We first carried out a MD simulation for the PLN+galardin complex and

analyzed the root mean square deviation (RMSD) from the initial structure. The RMSD is smaller than 0.1 Å, indicating that the initial structure of PLN+galardin is kept at 300 K. Next, the same MD simulation was conducted for the PLN+G6b complex to obtain the RMSD shown in Figure 10. Since RMSD fluctuates around 0.5 Å and is smaller than 0.7 Å, it was confirmed that PLN+G6b keeps its structure even at 300 K. It was also confirmed from the MD trajectories that G6b keeps staying at almost the same position in the ligand-binding pocket of PLN. Accordingly, it is concluded that G6b binds strongly to the ligand-binding pocket of PLN and can inhibit the PLN activity.

4. Conclusions

In the present molecular simulations, we investigated the binding properties between the metalloproteinase (PLN or MMP-9) and galardin derivatives, in order to propose novel compounds with higher binding affinity to PLN. The following points were elucidated by our *ab initio* FMO calculations and MD simulations.

(1) The BEs between galardin and MMP-9/PLN are comparable to the binding affinity observed by the previous experiment [9].
(2) The Zn ion and PLN residues around it are important for the binding of the derivatives.
(3) Among the derivatives proposed by the present study, G6b has the largest BE and keeps staying at the ligand-binding pocket of PLN to be a potent inhibitor against PLN.

Acknowledgements

This collaboration study is carried out under the official agreement of international collaboration study between Toyohashi University of Technology and the Arctic University of Norway and supported by the international internship program of Japan Student Services Organization (JASSO).

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