

Seeking mTORC1 Inhibitors Through Molecular Dynamics Simulation of Arginine Analogs Inhibiting CASTOR1

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Abstract. *Background:* Hyperactivity of the mechanistic target of rapamycin complex 1 (mTORC1) is implicated in a variety of diseases such as cancer and diabetes. Treatment may benefit from effective mTORC1 inhibition, which can be achieved by preventing arginine from disrupting the cytosolic arginine sensor for mTORC1 subunit 1 (CASTOR1)–GTPase-activating proteins toward RAGS subcomplex 2 (GATOR2) complex through binding with CASTOR1. An attractive idea is to determine analogues of arginine that are as competent as arginine in binding with CASTOR1, but without disrupting the CASTOR1–GATOR2 interaction. *Materials and Methods:* Molecular dynamics simulations were performed for binding of arginine analogues with CASTOR1 and binding free energy, hydrogen bond

formation, and root mean squared deviation and root mean square fluctuation kinetics were then calculated. *Results:* The binding free energy calculations revealed that N_{α} -acetyl-arginine, citrulline, and norarginine have sufficient binding affinity with CASTOR1 to compete with arginine. The hydrogen bond analysis revealed that norarginine, N_{α} -acetyl-arginine and D-arginine have proficient H-bonds that can facilitate their entering the narrow binding pocket. *Conclusion:* Norarginine and N_{α} -acetyl-arginine are the top drug candidates for mTORC1 inhibition, with N_{α} -acetyl-arginine being the best choice.

Mechanistic target of rapamycin (mTOR) in complex with regulatory-associated protein of mTOR (RAPTOR) and mTOR-associated protein, LST8 homolog (LST8) is referred as mTOR complex 1 (mTORC1). It is implicated in diseases in which growth is deregulated and homeostasis is compromised (1). Deregulated mTORC1 signalling fuels the destructive growth of cancer. Overstimulation of the mTORC1 pathway by excess food consumption may be a crucial factor underlying the diabetes epidemic (2-4). Recent findings suggest that mTORC1 signalling controls the rate at which cells and tissues age, and that inhibiting mTORC1 may represent a promising avenue for increasing longevity (5). In particular, dysregulation of signalling pathways upstream of mTORC1 can cause human diseases such as cancer, metabolic diseases, neurological disorders, and autoimmune diseases (6, 7).

In order for cells to grow and proliferate by manufacturing more proteins, the cells must ensure that they have the resources (energy, nutrients, oxygen, and growth factors) available in order for mRNA translation to begin (5, 8, 9).

This article is freely accessible online.

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Key Words: mTOR, CASTOR1, cancer, molecular dynamics simulation.

Nutrient availability is primarily sensed as the intracellular amino acid level, by the RAGULATOR–RAG complex sitting on the surface of the lysosome (10). Figure 1 uses leucine and arginine as examples to illustrate the pathway by which amino acids activate mTORC1. The RAGULATOR–RAG complex is formed by RAGULATOR and RAG GTPases, with the former serving as the scaffold of the latter. The RAG-GTPases form heterodimeric complexes comprised of RAGA or RAGB bound to RAGC or RAGD (11). To form the RAGULATOR–RAG complex, RAGA/B must be GTP loaded (12, 13) and RAGC/D must be GDP loaded (14, 15). This complex causes mTORC1 to be translocated from the cytoplasm to the lysosomal surface, which allows for the activation of mTORC1 by the lysosome-anchored small GTPase Ras homolog enriched in brain (RHEB) (13, 16).

GTPase-activating proteins toward RAGS subcomplex 1 (GATOR1) prevents RAGA/B from loading GTP and thus prevents the formation of the RAGULATOR–RAG complex and subsequent mTORC1 activation (17). To activate mTORC1, GATOR1 must be inhibited, and its upstream inhibitor is GATOR2. However, GATOR2 is usually associated with other proteins and is thus inactivated. These GATOR2-associating proteins are sensors for amino acids and there are a variety of them. For example, SESTRIN2 is a cytosolic leucine sensor (11) and CASTOR1 is an arginine sensor (6). These amino acids, when binding with their corresponding sensor proteins, can disrupt the association of the sensor proteins with GATOR2 (Figure 1). Released GATOR2 inhibits GATOR1 and ultimately leads to activation of mTORC1. For example, the amino acid leucine disrupts the GATOR2–SESTRIN2 complex and ultimately activates mTORC1 (11).

The present study focused on arginine, whose sensor is the protein CASTOR1. In order to associate with GATOR2, CASTOR1 needs to either homodimerize or heterodimerize with CASTOR2 (6), in a complex denoted by CASTOR1–GATOR2, which refers to either CASTOR1–CASTOR1–GATOR2 or CASTOR1–CASTOR2–GATOR2. Arginine, when present at sufficient concentrations, can bind to the conserved ACT domains of CASTOR1 and subsequently disrupts the CASTOR1–GATOR2 complex (18, 19). As a consequence, GATOR2 dissociates from CASTOR1–homodimer or CASTOR1–CASTOR2, leading to the ultimate activation of mTORC1 (6, 11).

Given that mTORC1 hyperactivation underlies complex diseases such as cancer and diabetes, this study considered methods of mTOR inhibition. From the above, it is clear that maintenance of CASTOR1–GATOR2 association is key to inhibition of mTORC1. That is, as long as GATOR2 is associated with either CASTOR1–CASTOR1 or CASTOR1–CASTOR2, it has no opportunity to activate the downstream mTORC1. Therefore, the key to preventing mTORC1

hyperactivation is to maintain integrity of the CASTOR1–GATOR2 association. This association is, however, difficult to maintain, because arginine is ubiquitously present in cells, which can easily bind with CASTOR1 and subsequently disrupt the CASTOR1–GATOR2 association. It is thus highly desirable to develop small-molecule drugs that can occupy the binding pocket of CASTOR1 to arginine and still maintain integrity of the CASTOR1–GATOR2 interaction. Naturally, arginine analogues are potential drug candidates for fulfilling the above requirements, namely, to target the conserved ACT domain of CASTOR1 for the treatment of diseases related to mTORC1 hyperactivation.

The arginine analogues reported by Saxton *et al.* (18) drew our attention. These arginine analogues can be divided into two groups (Figure 2): those that can disrupt the CASTOR1–GATOR2 interaction (called the disruption group) and those that cannot (called the non-disruption group). The disruption group includes L-arginine, canavanine (a non-proteinogenic amino acid with the sole difference from arginine being the replacement of a methylene bridge in arginine with an oxygen atom in canavanine), and a carboxy-modified arginine-methyl ester (arginine-OMe). The non-disruption group includes compounds with alterations to the guanidinium group, α -amine, or the length of the side chain (18). In this study, the interactions between these arginine analogues and CASTOR1 were studied at the atomic level by molecular dynamics simulation (MD simulation) in order to provide insights into development of drug candidates targeting mTORC1 hyperactivation. Specifically, we aimed to select inhibitors from the non-disruption group that are sufficiently competitive with arginine in terms of binding with CASTOR1.

Materials and Methods

Structure preparation. CASTOR1 protein, arginine and arginine analogues, water and ions were used to construct the simulation system. The atomic coordinates of CASTOR1 were obtained from the crystal structure 5I2C, which was an arginine bound human CASTOR1 structure (20). To obtain the pure CASTOR1 structure, arginine and water molecules were removed and the missing amino acids were then complemented by homology modelling. The protein was then minimized and equilibrated in a water box with salt for 1000 ns by MD simulation. The 10 arginine analogues (Figure 2) were then prepared *in silico*. They were drawn by Discovery Studio 2016 client software (BIOVIA, San Diego, CA, USA). They were then respectively docked to the CASTOR1 structure to start MD simulation.

Simulation methods. MD simulation is a powerful method for studying the kinetics of protein–ligand binding (21, 22). Molecular docking, MD simulation, and free energy calculation methods were used in this work. Molecular docking of each small-molecule drug to the active site of CASTOR1 was performed by AutoDock Vina (The Scripps Research Institute, San Diego, CA, USA) (23). Docking simulations not only provide the binding affinity data but

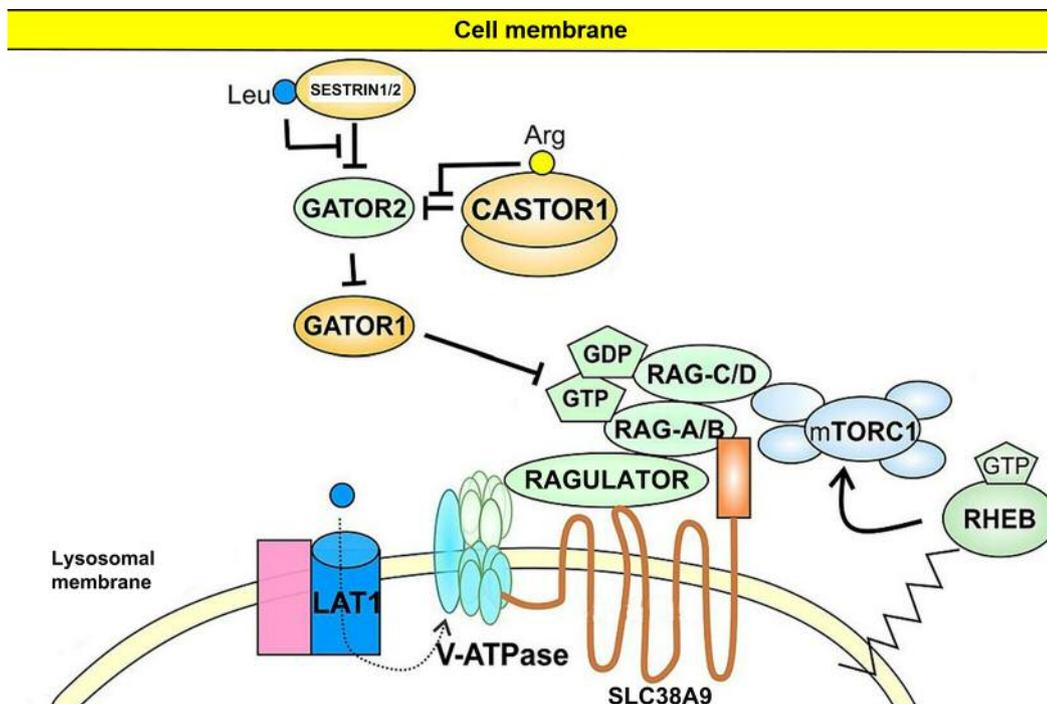


Figure 1. Amino acid signalling through cytosolic arginine sensor for mechanistic target of rapamycin complex 1 (mTORC1) subunits (CASTORs), SESTRINS, and solute carrier family 38 member 9 (SLC38A9) to regulate mTORC1 activity. In particular, binding with CASTOR1 (an arginine sensor) is required for arginine to activate the mTORC1 pathway. GATOR1/2: GTPase-activating proteins toward RAGS subcomplex 1/2; RHEB: Ras homolog enriched in brain; LAT1: linker for activation of T-cells.

also obtain the optimized holo-system for the subsequent MD simulation. Ten simulation boxes were then built to simulate the MD of the 10 small molecules interacting with CASTOR1. Before the MD simulation, TIP3P water model (24) was employed to build the water box and some chlorine or sodium ions were added to neutralize the system. AMBER16 package (25) was employed to run all the MD simulations with leaprc.protein.ff14SB (26) as the force field for the protein, and General Amber Force field (GAFF) (27) as the force field for the arginine analogues.

Simulation protocol. For each simulation box, energy minimization was first performed to obtain a low-energy starting conformation for the subsequent MD simulations. Four-thousand steps of steepest descent method was first employed followed by six-thousand steps of conjugate gradient method. The whole system (protein, ligand, water, ions) was first minimized, followed by minimization on the solutes (protein and ligand) only. With the Langevin thermostat applied, the system was heated under canonical ensemble from 0 to 303 K for 300 ps, with the force constant for the harmonic restraint set at $10.0 \text{ kcal mol}^{-1} \text{ \AA}^{-2}$. The system was then equilibrated for 10 ns under isothermal-isobaric conditions (with constant pressure of 1.0 bar). The relaxation time for the barostat bath was set at 2.0 ps. Finally, the production simulation was run for 100 ns under isothermal-isobaric conditions with periodic boundary conditions. The time step was set at 2 fs and bonds connected with hydrogen atoms were constrained using the SHAKE algorithm. The long-range electrostatics was handled by the particle-mesh Ewald method

(28). The cut-off value for short range interactions was set at 10.0 \AA . The production simulation was repeated three times in order to calculate the average.

Binding free energy calculation. The free energy of CASTOR1 binding to arginine analogue was calculated by the molecular mechanics energies combined with the generalized born and surface area continuum solvation method (29-32). In order to identify the most crucial residues of CASTOR1 for the binding of the arginine analogue, the total binding free energy was decomposed into contributions from individual residues ($i=1, 2, \dots, 342$):

$$\Delta G_{\text{bind}} = \sum_{i=1}^{342} \Delta G_{\text{bind}}^i = \sum_{i=1}^{342} \sum_{j \neq i}^{342} \Delta G_{\text{bind}}^{ij}$$

where ΔG_{bind}^i were the per-residue contributions, and $\Delta G_{\text{bind}}^{ij}$ were the residue-pairwise interaction contributions. The calculations were rendered by the MMPBSA.py.MPI module (33) of AMBER16.

Results

Root mean square deviation (RMSD) and root mean square fluctuation (RMSF). MD simulations were performed on the 10 arginine analogue systems to reveal the overall dynamical

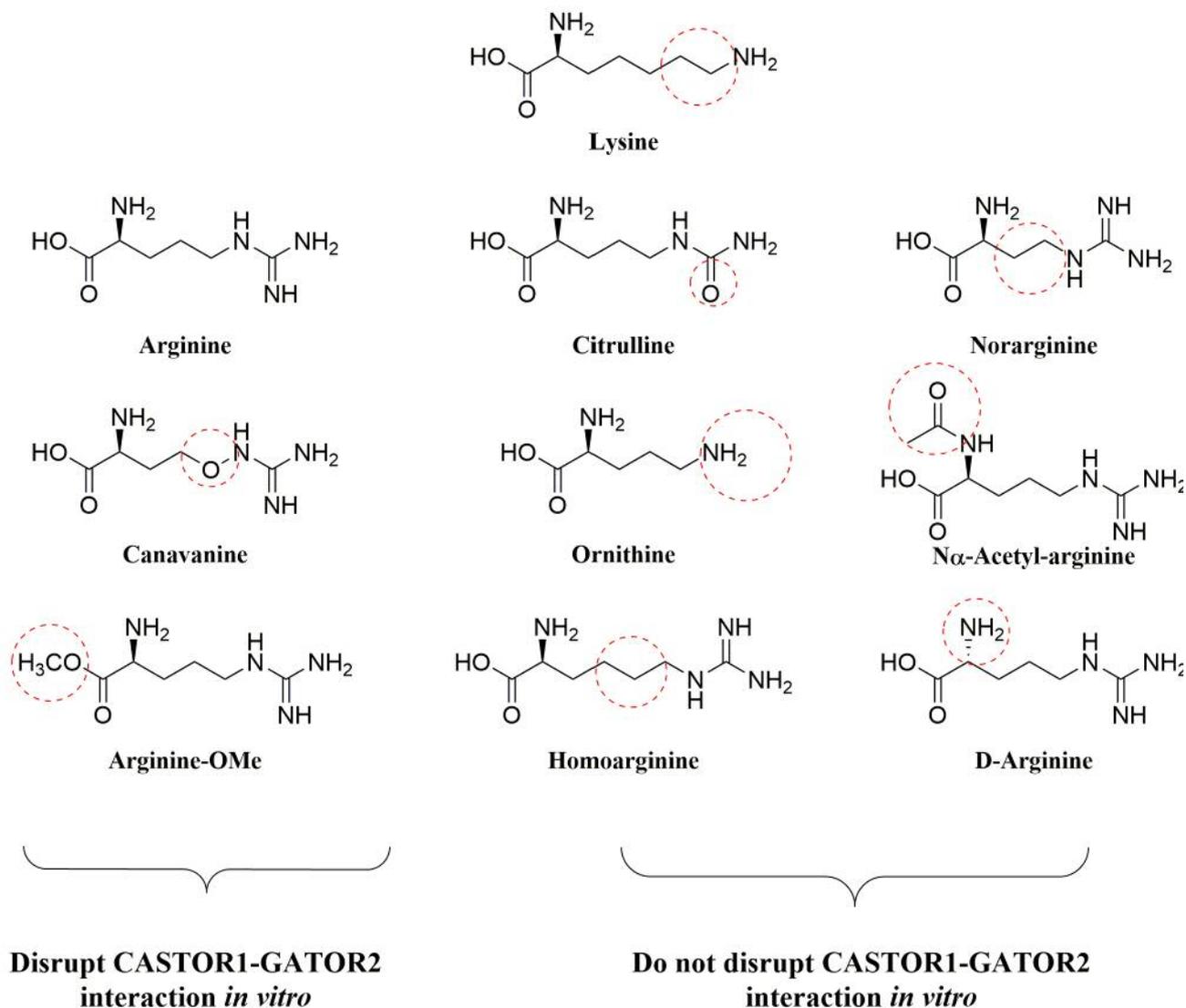


Figure 2. Chemical structures of arginine and its analogues. Differences from arginine are highlighted by dashed circles.

features of the binding between CASTOR1 and arginine analogues, and to determine the difference between the disruption and the non-disruption groups. The calculation of RMSD can be used to investigate the variation and stability of protein–drug complex. The disruption group consisted of three arginine analogues (arginine, canavanine, and arginine-OMe) and their RMSD results are presented in Figure 3A. It can be seen that the initial unsteady state lasted about 20 ns before the atoms stably oscillated around their new positions. For the L-arginine system, the new position was about 3.75 Å away from the initial position during 20 to 80 ns, after which the atoms moved a little further away (4 Å). For the canavanine and arginine-OMe systems, the new position was about 2.75 Å away from the initial position during 20 to 80 ns, after which

the atoms also moved a little further away (3 Å). The non-disruption group consisted of seven arginine analogues and their RMSD results are presented in Figure 3B. Except D-arginine, the atoms behaved similarly to those of the disruption group, oscillating around a new position some 3 Å away from the initial position. The D-arginine system atoms fluctuated considerably between 30 ns and 70 ns before finally stabilizing 3.75 Å away from the initial position. This difference appeared to be attributed to the chiral carbon distinguishing D-arginine from arginine. These results demonstrate that arginine and D-arginine exerted much stronger influence on protein conformation than the other arginine analogues.

RMSF analysis was then used to estimate the fluctuations of each amino acid residue over the simulation time. We

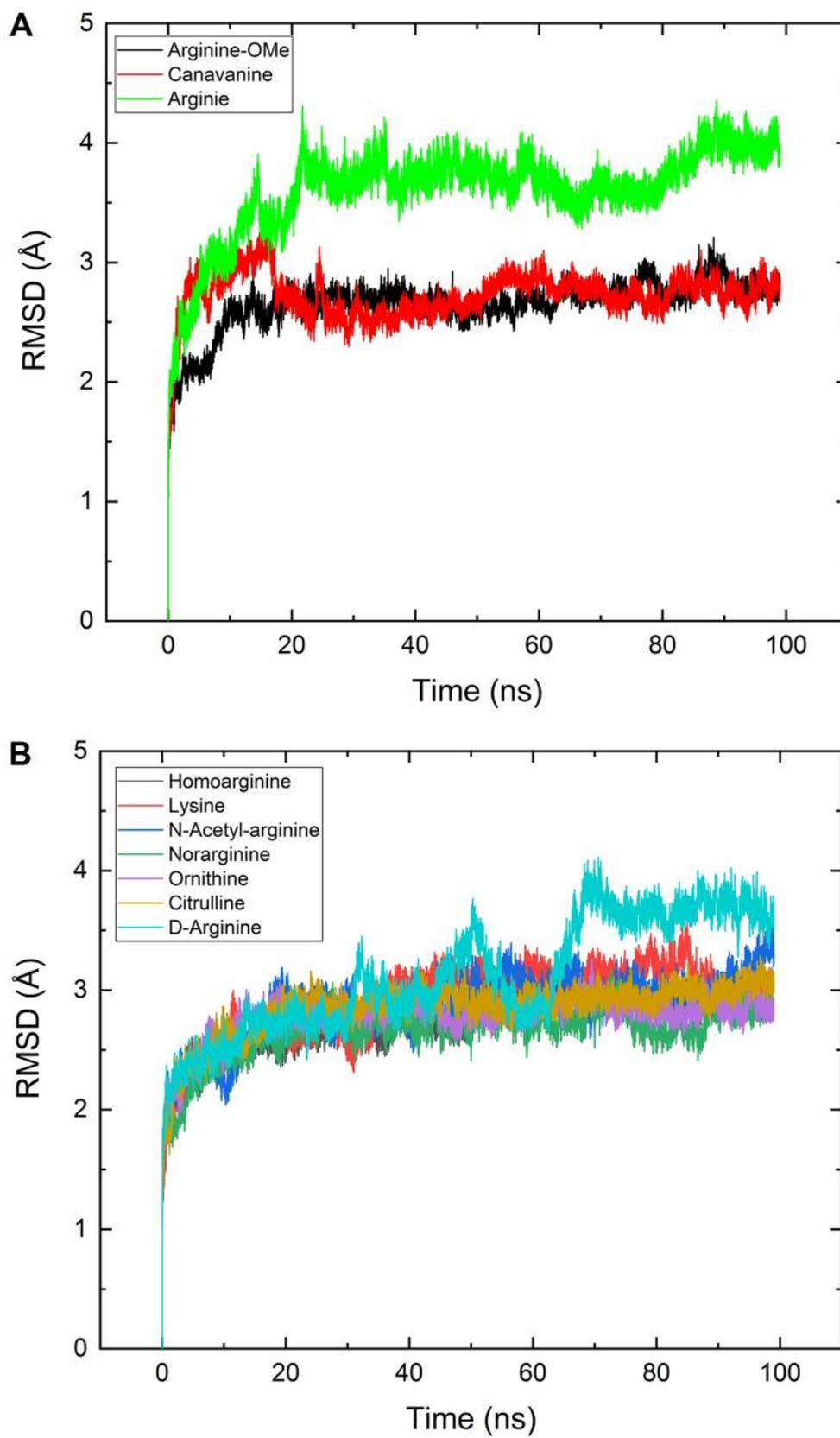


Figure 3. Time course of root mean square deviation (RMSD) values for the disruption (A) and non-disruption (B) groups of arginine analogues.

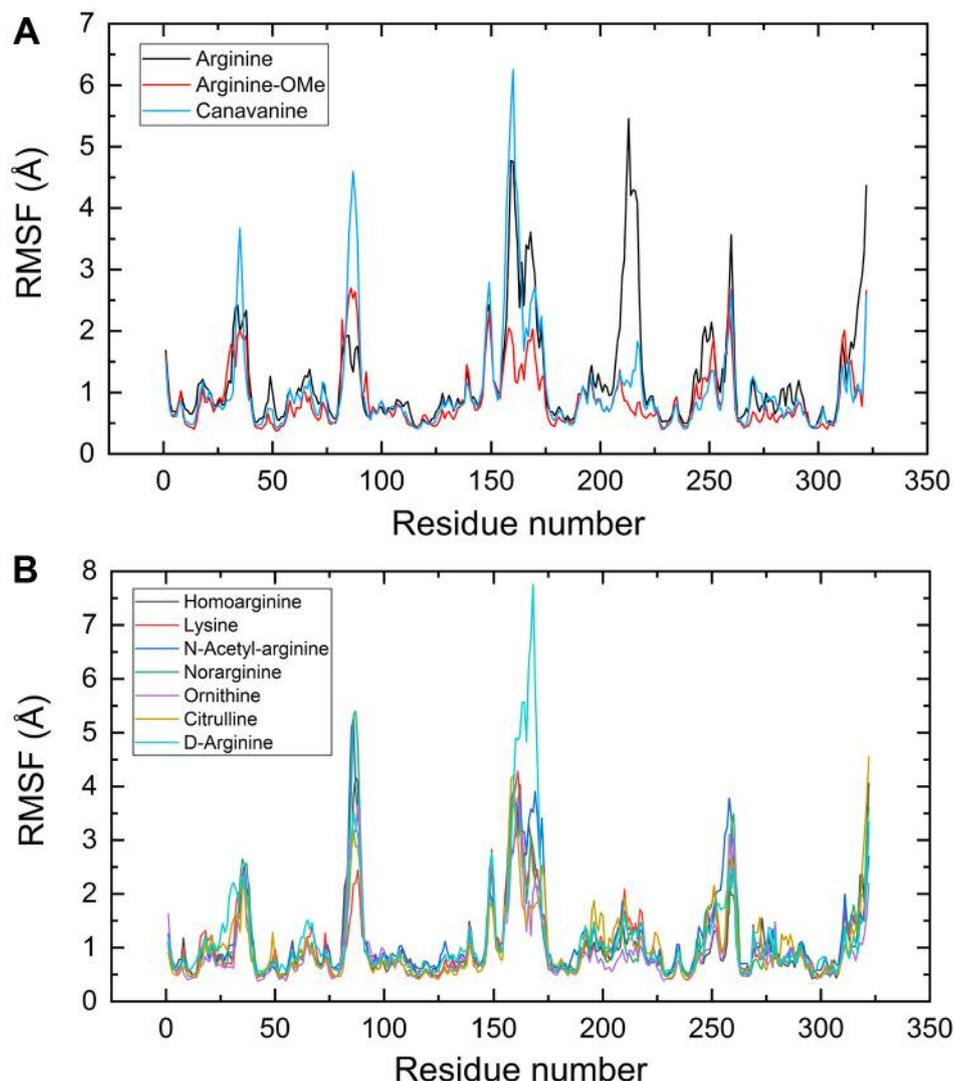


Figure 4. Time course of root mean square fluctuation (RMSF) values for the disruption (A) and non-disruption (B) groups of arginine analogues.

found that most of the systems fluctuated considerably around amino acids 25-50, 75-100, 150-175, 250-275 and 300-325 (Figure 4). Note that these residues belong to the domains ACT2 and ACT4, which concurs with the fact that CASTOR1 bound to arginine through a narrow pocket at the interface of ACT2 (amino acids 75-156) and ACT4 (amino acids 259-329) (1). Therefore, the differences of the arginine analogues from arginine do not alter their approximate docking site on CASTOR1. However, there was one significant difference between arginine and its analogues. In the arginine system, residue Lys213 of CASTOR1 had the highest RMSF value, indicating its large fluctuation. Surprisingly, Lys213 does not locate on the interface of ACT2 and ACT4.

Table I. Summary of binding free energy for arginine analogues with cytosolic arginine sensor for mechanistic target of rapamycin complex 1 subunit 1.

	Analogue	ΔG_{bind} (kcal mol ⁻¹)
Disruption group	Arginine	-44.6420
	Canavanine	-33.3440
	Arginine-OMe	-39.7628
Non-disruption group	Citrulline	-31.5504
	Norarginine	-29.7002
	Ornithine	-17.0416
	Homoarginine	-16.0391
	N _α -Acetyl-arginine	-36.2542
	Lysine	-20.1681
	D-Arginine	-17.0416

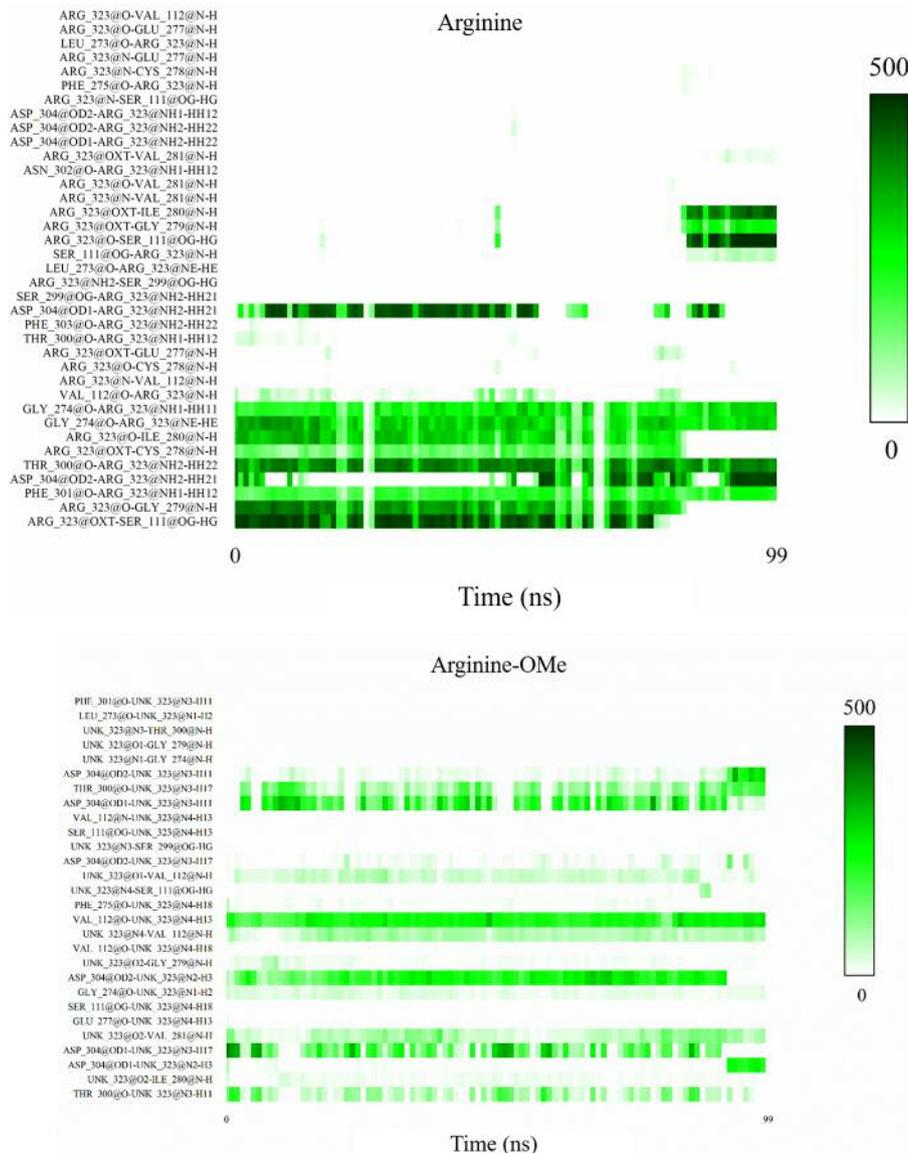


Figure 5. Continued

Binding free energy analysis. Affinity of enzyme–substrate binding can be estimated by calculating the free energy of binding ΔG_{bind} . In this study, the last 20 ns of the MD simulation was used to calculate the binding free energy by using the MM-GBSA method. With the lowest binding free energy ΔG_{bind} of $-44.6420 \text{ kcal mol}^{-1}$, the arginine system was the most stable complex among the 10 systems (Table I). Moreover, the binding of molecules of the disruption group with CASTOR1 was generally more stable than that of the non-disruption group, which demonstrates that our choice of drug candidates would not be abundant. Nevertheless, N_{α} -acetyl-arginine in the non-disruption group had ΔG_{bind} of $-36.2542 \text{ kcal mol}^{-1}$, which was the third

lowest binding free energy and was even lower than that of canavanine in the disruption group. Therefore, N_{α} -acetyl-arginine might be a strong candidate if other measures (such as hydrogen bonding; see below) are also suitable. Besides N_{α} -acetyl-arginine, citrulline and norarginine in the non-disruption group also had very low binding free energy with CASTOR1 (around $-30 \text{ kcal mol}^{-1}$). Therefore, N_{α} -acetyl-arginine, citrulline, and norarginine may be competitors of arginine and are thus potential drug candidates.

Hydrogen bond (H-bond) analysis. H-Bond analysis is important in structure-based drug design. In general, the more H-bonds formed, the more stable the interaction. If an

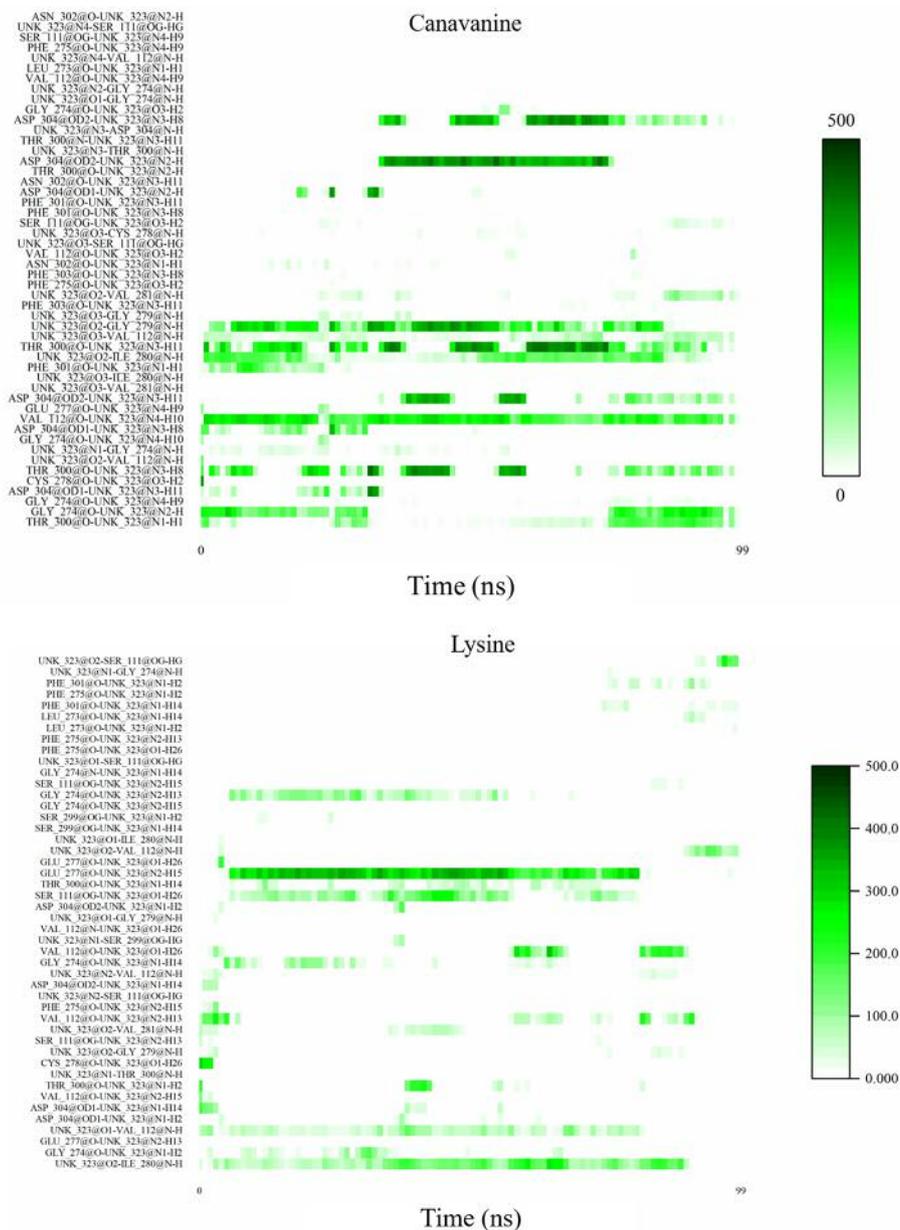
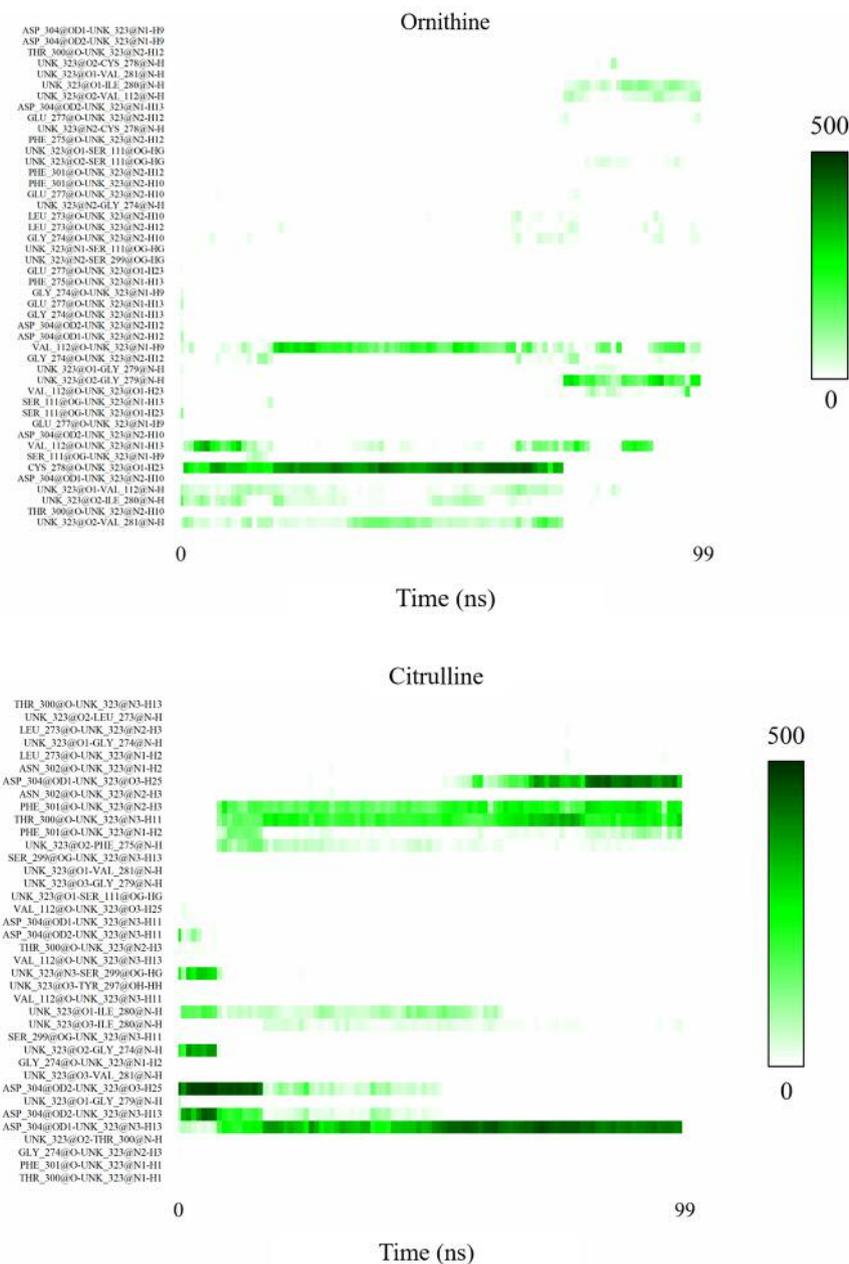


Figure 5. Continued

inhibitor in the non-disruption group forms significantly more H-bonds with CASTOR1 than arginine does, then it would be a good candidate mTORC1 inhibitor. We therefore performed a comprehensive H-bond analysis of the 10 systems. During the 100 ns MD simulation of each system, the real-time formation of H-bonds was monitored, and the results are presented in Figure 5. To comprehend more intuitively, the structure of the binding pocket formed by the ligand and its surrounding amino acid residues is illustrated, with the primary H-bonds indicated by green dashed lines (Figure 6).

We first analysed the disruption group. For arginine, its C-terminus and N-terminus mainly form H-bonds with nearby CASTOR1 residues such as Ile280, Gly279, Cys278, Val112, and Ser111. At the other end, the guanidine group interacts with Gly274, Asp304, Thr300, and Phe301. The molecule arginine-Ome is derived from arginine by replacement of the carboxyl group with an ester group, which causes a small rotation abolishing the H-bond between the C-terminus and Cys278 and generating a new H-bond between the C-terminus and Val281. The rotation also abolishes the H-bond between the N-terminus and Phe301 and generates a new H-

Figure 5. *Continued*

bond between the *N*-terminus and Ser299. Canavanine is derived from arginine by replacing *C*-5 with an oxygen atom, which reduces H-bond availability on Ser111 and Cys278. It is clear that arginine-OMe and canavanine do not differ significantly from arginine as far as H-bonding is concerned. The results demonstrate consistency of the MD simulation and H-bond analysis for the three molecules.

In the non-disruption group, we first analysed lysine, ornithine, and citrulline, which are derived from arginine by replacing the guanidine group with three other groups. This

replacement does not enhance H-bonding because the lost guanidine group is an important H-bond donor. Given that the replacements are more thorough in lysine and ornithine (from the guanidine group to N2H), the two analogues would have greater loss of H-bonds. Indeed, lysine and ornithine fail to form important H-bonds with Phe301 and Asp304 due to the lack of two H-bond-donating nitrogen atoms in the guanidinium group. For citrulline, the guanidine group is replaced by a bio-isostere, and the substitution of oxygen for imidogen provides a new H-bond interaction between the O

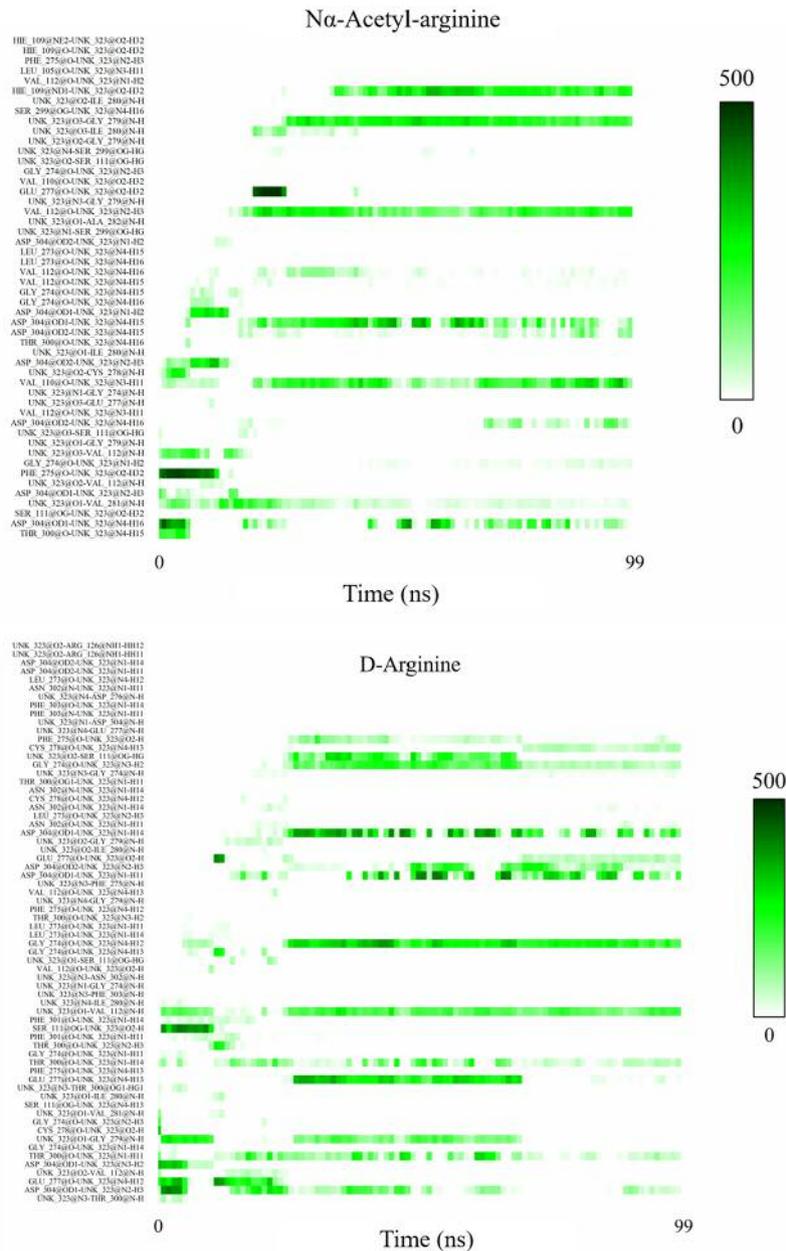


Figure 5. Frequency of hydrogen bond formation between arginine analogues and surrounding amino acid residues of cytosolic arginine sensor for mechanistic target of rapamycin complex 1 subunit 1. Each row corresponds to a specific hydrogen bond. The columns correspond to time zones dividing the 100 ns simulation time. The colours indicate the times of H-bond formation: the darker the colour, the more frequent the H-bond formation.

found that both N_{α} -acetyl-arginine and D-arginine form more H-bonds than does the arginine system.

The H-bond analysis revealed that norarginine, N_{α} -acetyl-arginine and D-arginine are competitive with arginine in binding with CASTOR1. Unlike arginine, which disrupts the CASTOR1–GATOR2 complex, the three analogues belong to the non-disruption group and are thus potential drug candidates for mTORC1 inhibition.

Discussion

mTORC1 hyperactivation causes many complex diseases such as cancer, neurological disorders, metabolic diseases, and autoimmune diseases. Therefore, mTORC1 inhibition is a potentially effective therapeutic strategy. mTORC1 inhibition can be realized by maintaining integrity of the CASTOR1–GATOR2 complex *in vivo*. Because arginine

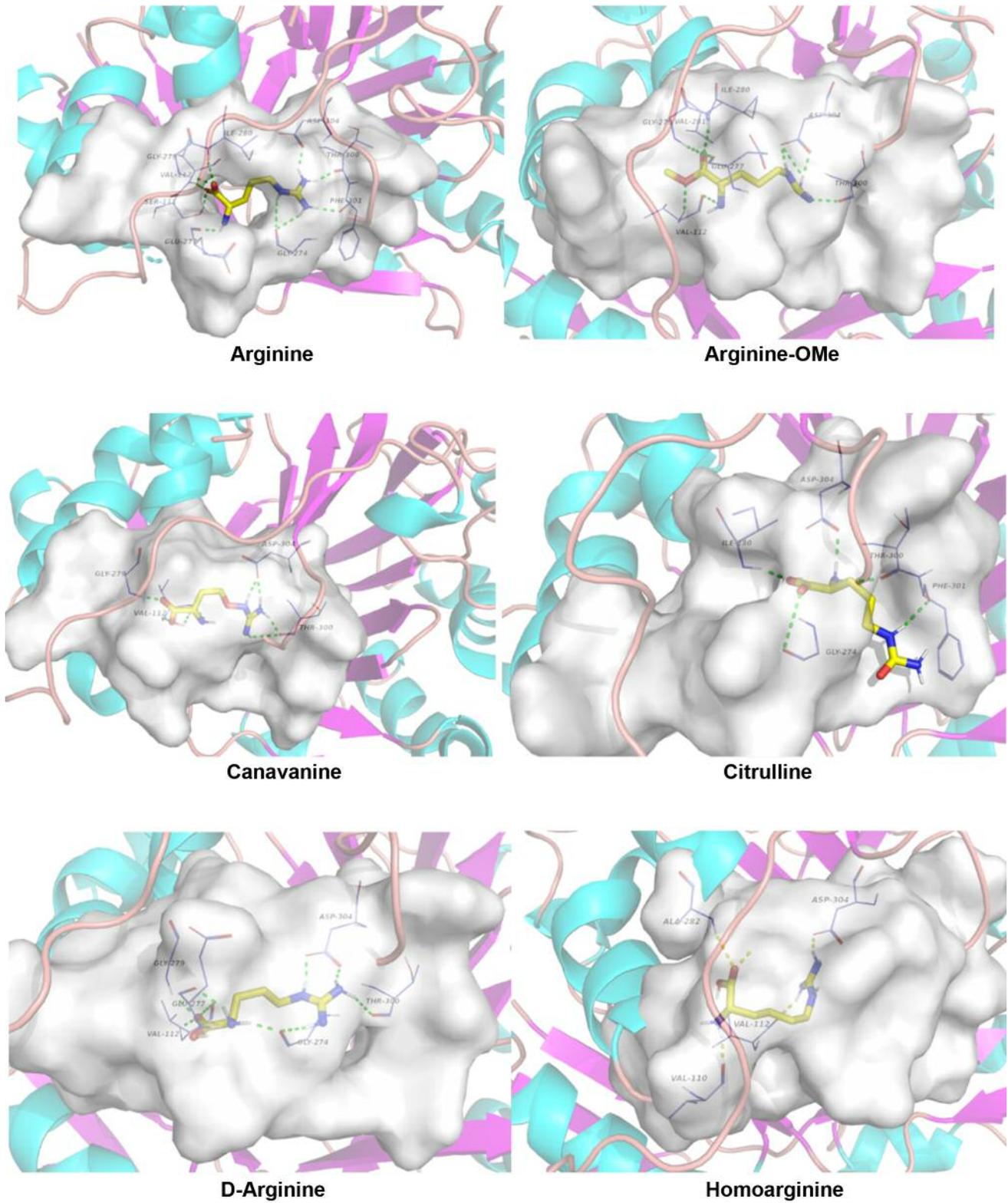


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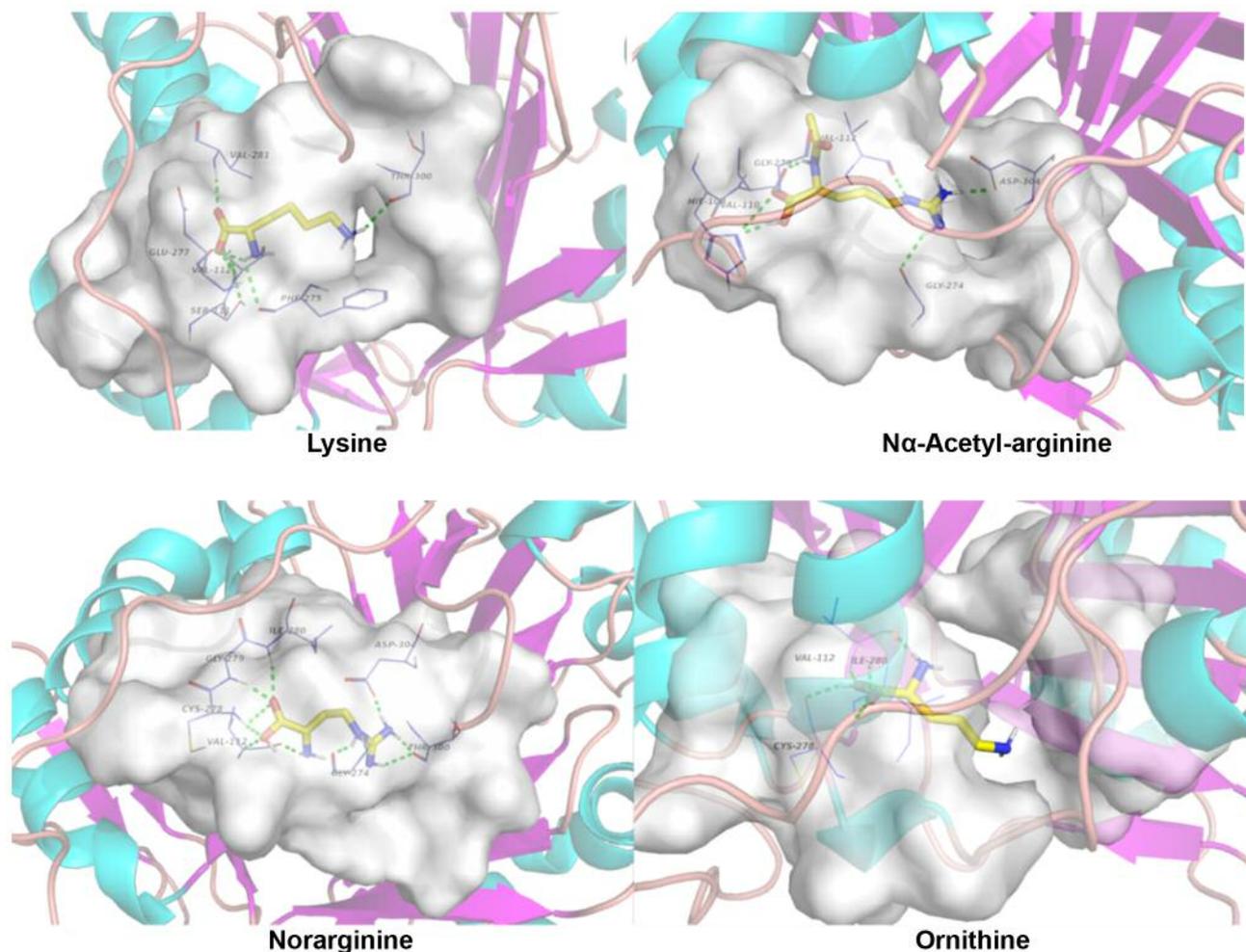


Figure 6. Structure of the pocket of binding between arginine analogues and cytosolic arginine sensor for mechanistic target of rapamycin complex 1 subunit 1. The hydrogen bonds are indicated by green dashed lines.

can bind with CASTOR1 and disrupt the CASTOR1–GATOR2 complex, it is crucial to prevent arginine from binding with CASTOR1. An attractive idea is to find arginine analogues that are as competent as arginine in binding with CASTOR1 but do not disrupt the CASTOR1–GATOR2 interaction.

In this study, we performed MD simulation of the interaction of arginine analogues with the protein CASTOR1. The RMSD and RMSF analyses revealed key features of the atomic level dynamics, which agree with the structural features of the protein–ligand interaction, thus demonstrating the validity of our approach. The analyses also highlighted the differences in binding dynamics among arginine analogues. The binding free energy calculations revealed that N_{α} -acetyl-arginine, citrulline, and norarginine have sufficient binding affinity with CASTOR1 to compete

with arginine. The H-bond analysis revealed that norarginine, N_{α} -acetyl-arginine and L-arginine have proficient H-bonds that can facilitate their entering the narrow binding pocket of CASTOR1. We therefore suggest that norarginine and N_{α} -acetyl-arginine are the top drug candidates for testing experimentally and clinically. Moreover, N_{α} -acetyl-arginine is the best choice because its binding affinity with CASTOR1 is very high, only lower than arginine and arginine-OMe. Importantly, N_{α} -acetyl-arginine forms more hydrogen bonds with CASTOR1 than arginine, which should confer additional advantages in binding with CASTOR1.

Conflicts of Interest

The Authors declare no conflicts of interest in regard to this study.

Authors' Contributions

GW, WS, GRFK designed research; LS and XL performed research; LS, XL, JP, JM, YY, DW, WS, GRFK, GW analysed data; LS, XL, DW, and GW wrote the article. All Authors read and approved the final article.

Acknowledgements

This work was partly supported by National Natural Science Foundation of China (61773196), Guangdong and Shenzhen Research Funds (JCYJ20170307104535585 and JCYJ20170817104740861, ZDSYS20140509142721429, 2017B030301018, 2019B030301001), Shenzhen Peacock Plan (KQTD2016053117035204), and by Center for Computational Science and Engineering of Southern University of Science and Technology.

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Received July 15, 2019

Revised July 25, 2019

Accepted July 30, 2019