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COMPUTATIONAL MODELLING OF TRANS-ZEATIN AS A NOVEL TARGET OF ADENOSINE A2A RECEPTOR: INSIGHTS INTO MOLECULAR INTERACTIONS

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Ethics committee approval: There is no need for ethics committee approval.

Abstract

Adenosine A2A receptor (A2AR) is a G-protein coupled receptor that is involved in various physiological functions. Zeatin, a plant cytokinin and a derivative of adenine, is recently identified as new ligand of A2AR. However, the ligand-receptor interaction mechanism is not fully revealed. Here, we report a model structure of A2AR in complex with zeatin for the first time, to provide a better understanding of this interaction mechanism. A model structure of A2AR in complex with caffeine used as a positive control. As a result, zeatin displayed the ability to stay more stable at the binding pocket compared with caffeine and the residues involved in the interaction are identified. We propose that zeatin is indeed a novel and promising target for A2AR

Keywords: adenosine A2A receptor, binding pocket, caffeine, protein-ligand interaction, zeatin

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1. Introduction

Adenosine receptors (A1, A2A, A2B, A3) are transmembrane G-protein coupled receptors (GPCRs) that can either stimulate or inhibit adenylyl cyclase (AC) by their Gi and Gs subunits (Lazarus et al., 2011). Adenosine A1 receptor (A1R) and adenosine A3 receptor (A3R) inhibit the AC via Gi subunit,whereas adenosine A2A receptor (A2AR) and adenosine A2B receptor (A2BR) stimulate the activity of AC via Gs subunit, catalyze the production of cyclic adenosine monophosphate (cAMP) (Thiel., 2003). A2ARs are expressed in higher density in the basal ganglia of brain and in lower density in the cardiovascular, and immune system (Schiffmann et al., 2007; Doré et al., 2011). A2AR consists of seven transmembrane domains with an extracellular amino terminus and a cytosolic carboxy terminus as a common feature with all GPCRs. The connection between transmembrane domains consist of between three extracellular and three cytoplasmic loops (Ijzerman et al., 1994). The binding of a ligand occurs on the extracellular side, leading to the conformational changes in the heptahelical transmembrane helix network of the receptor (Jaakola et al., 2010).

Caffeine is a plant-derived methylxanthine and a well known nonselective antagonist of A2AR (Fredholm et al., 2005). A2AR has well defined binding pocket and previous studies showed that caffeine interacts with the residues PHE 168, ILE 274, LEU 249, MET 270, ASN 253, TRP 246, and VAL 84 with an additional polar contact to HIS 278 in the hydrophobic pocket (Dore et al., 2011; Carpenter et al., 2017). Caffeine blocks the activity of A2AR and therefore, it is considered as an effective and widely consumed psychoactive drug and stimulant (Fredholm et al., 1995). Caffeine can display neuroprotective effects by preventing the β-amyloid-induced neurotoxicity or promote wakefulness. (Lazarus et al., 2011; Dall'Igna et al., 2003; Dall'Igna et al., 2007).

Zeatin,a plant phytohormone, promotes plant growth and development, was recently shown to interacts with A2AR, however the binding mechanism has not been discovered yet (Lee et al., 2012). Plant cytokinins are adenine derivatives substituted at the N6-position with either an isoprenoid or aromatic side chain, and cis- and trans-zeatin includes a substitiution of isoprenoid side chain (Lee et al., 2012). Cytokinins have important antioxidative and protective effects in animals at molecular, cellular, tissue and organismal levels (Voller et al., 2017). Zeatin showed antioxidative and cell protective effects against β-amyloid-induced neurotoxicity, similar to caffeine (Choi et al., 2009). Most recently, the possible anti-depressant effect of zeatin on female and male rats was shown, together with the interaction of zeatin with A2A receptor on the same binding site with caffeine (Öz et al., 2020). These findings further suggest that zeatin exerts its effects via A2A-R-mediated downstream pathways. Zeatin can be converted to zeatin riboside by adenosine phosphorylase and zeatin riboside was shown to prevent the serum deprivation-induced apoptosis (Dall'Igna et al., 2007). Previously, it has been known that A2ARs regulates CD4+ T lymphocyte, even suppressing the activation-induced cell death (AICD) of peripheral T cells (Himer et al., 2010). In addition to, zeatin riboside treatment promotes the production of cAMP in T lymphocytes and inhibits the production in CD3+CD4+ T cells of interferons (Lappas et al., 2015). These findings clearly indicate an interaction between zeatin and A2AR, and better understanding of this interaction can be achieved by structural modelling.

2. Materials and Methods

The authors declare that ethics commitee approval does not required for this study, as the study involves only computational work.

2.1. Structure preparation and docking

The crystal structure of A2AR (PDB entry: 5NLX) was used for docking of the ligands: trans-zeatin and caffeine. All other ligands found in the crystal structure of A2AR were removed in order to exert protein for docking step via PyMol (DeLano et al., 2002). Trans-Zeatin and caffeine were used as the ligand while A2A was kept as the receptor. Structures of ligands were obtained from RCSB PDB database and its parameters were determined using SwissParam (Zoete et al., 2011). The crystal structure of A2AR that contains caffeine as a ligand (PDB entry: 5MZP) was used as a control during docking to detect possible binding pocket and connecting residues based on previously published alignments (Carpenter et al., 2017; Cheng et al., 2017). The docking position of caffeine and trans-zeatin were detected in the same binding pocket and docked structure of A2AR complex with caffeine used as positive control during the study. Docking was performed with AutoDock (Goodsell et al., 1996). Final docking poses were selected based on Autodock binding scores that gives best down binding energy and inhibition constant.The binding interactions that were performed during docking were analyzed by Arpeggio (Jubb et al., 2017) and PyMOL(DeLano et al., 2002).

2.2. Designing of the membrane bound protein

Membrane protein tutorial (Aksimentiev et al., 2009) was performed to docking structures A2AR in complex with zeatin (5NLX/ZEA) and A2AR in complex with caffeine (5NLX/CFF) ,were produced previously via AutoDock (Goodsell et al., 1996), by using Visual Molecular Dynamics (VMD) (Humphrey et al., 1996). To provide interaction between membrane and protein, the following steps were adapted from membrane protein tutorial (Aksimentiev et al., 2009): generating PSF and PDB files for builting whole structure, building membrane patch for preparing complete membrane involving water around it, alignment of membrane and protein, combination of membrane and protein for avoiding overlap between protein and lipid molecules, solvation and ionizaiton with 100mM NaCI as the ionic concentration of the system. To perform molecular dynamic simulations, the structures were fixed according membrane protein tutorial (Aksimentiev et al., 2009).

2.3. Molecular dynamics simulations

Molecular dynamics (MD) simulations were performed with the structures that were obtained as a result of the previously described in the stage of desingning of membrane bound protein. The 5NLX/ZEA and 5NLX/CFF complex, composed of 78892 atoms and 78757 atoms were placed in water boxes with dimensions of 83x94x120 Å3 and neutralized with NaCl. The resulting systems for 5NLX/ZEA and 5NLX/CFF were used in MD simulations by

using the NAMD Phillips et al. (2005) with the CHARMM22 MacKerell et al. (1998); Brooks et al. (2009) parameters included correction map (CMAP) for backbone atoms (Feig et al., 2003; MacKerell et al., 2004). TIP3P model Jorgensen et al. (1983) was used for water molecules within the system during the simulation. An NpT ensemble was used in MD simulations with periodic boundary conditions to maintain pressure and temperature while the long-range Coulomb interactions were computed using the particle-mesh Ewald algorithm. Within 50 ns of MD simulation, the pressure was maintained at 1 atm and temperature was maintained at 310 K using the Langevin pressure and temperature coupling. The time step was determined as 1 fs in all MD simulations. For providing the removal of high energy contacts between atoms and highly repulsive orientations of the initial simulated systems, the systems were fully energy minimized in 50,000,000 steps and each system was heated slowly from 0 K to 310 K in 5 ps. Then, the systems were equilibrated under constant temperature and volume for 0.5 ns before production runs. The production runs were completed for 50 ns as it is a large complex and repeated twice. In total, 300 ns MD simulation was performed during the study.

2.4. Detection of interacting residues with ligands: zeatin and caffeine

The MD simulations which were repeated twice as previously described by using the NAMD Phillips et al. (2005) were analyzed to detect interacting residues between protein (5NLX) and ligands (zeatin and caffeine) by using VMD (Humphrey et al., 1996). Hydrogen bonds, Hydrophobic contacts and salt bridges were analyzed in VMD (Humphrey et al., 1996) for the detection of interacting residues during the simulations. Then, these interactions were plotted by using GraphPad Prism (Miller et al.,2003). For the comparison of detected residues with published alignments, a known A2AR with caffeine (PDB entry:5MZP) was used and the residues of 5MZP were renumbered as protein sequence was numbered as 1-305 in 5MZP while protein sequence was numbered as 9-409 in our model.

2.5. Analysis of the molecular dyanmics simulations

VMD (Humphrey et al., 1996) was used for the analysis of trajectories and the visualization of structures. Root mean square displacements (RMSDs) for the backbone atoms of each protein were analyzed for the stability in 50 ns MD simulations. Residue-specific distance between ligand (zeatin and caffeine) and protein was analyzed within 50 ns. In addition, Distance between the interacting atom of residue and the interacting atom of ligand (zeatin and caffeine) was analyzed. Residue-wise root mean square fluctuations (RMSFs) of ligands with protein were measured for the flexibility analysis of the ligand and t-test was applied to determine the significant difference between zeatin and caffeine values based on p-value of <0.05. The numerical data was expressed as mean ± SEM in the graph. RMSFs of the detected residues of the protein were also analyzed within 20-50 ns based on the result of distance analysis because the time that ligands are unstable was detected by interpreting distance and RMSF was calculated within this range. Also, Radius of gyration was analysed for both 5NLX/ZEA and 5NLX/CFF complex. Distance, Radius of Gyration and RMSF graphs were plotted by taking the average of 3 production runs for both 5NLX/ZEA and 5NLX/CFF complex via GraphPad Prism Jorgensen et al. (1983) while RMSD graphs were plotted for each production run respectively via GraphPad Prism (Miller et al.,2003).

3. Results

Trans-zeatin and caffeine were docked to the binding pocket of A2AR (PDB entry:5NLX) as described (Figure 1A) and the docking structure 5NLX/CFF was used as the positive control during this research to understand this targeting mechanism.

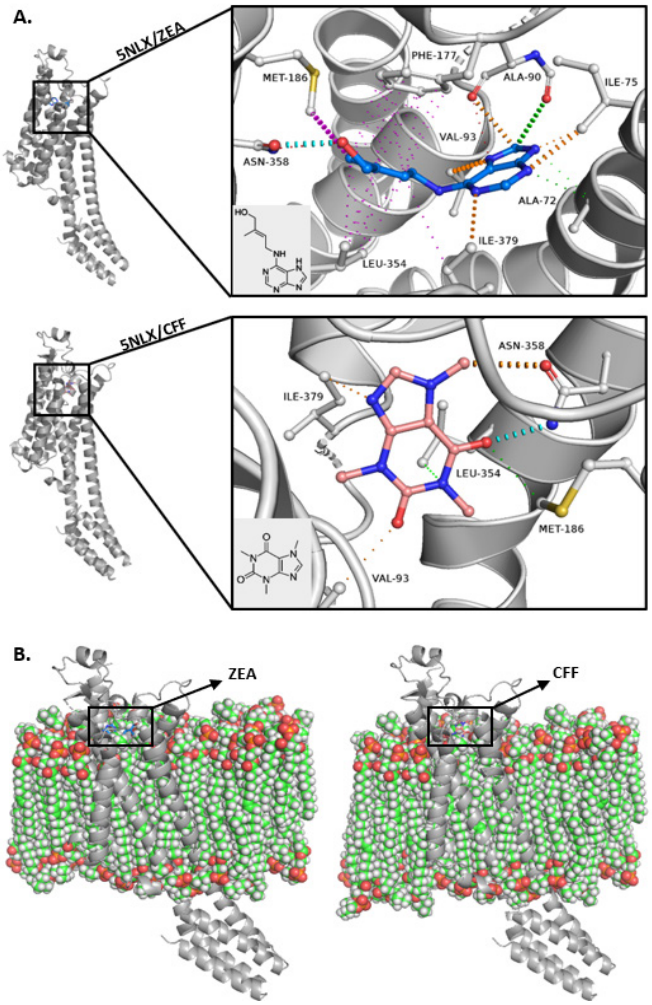

Figure 1: Detection of protein-ligand interaction and membrane alignment before the MD simulations.

A. Interaction of A2AR with ligands (zeatin and caffein) after docking were detected using Arpeggio (Jubb et al., 2017) and PyMOL (DeLano et al., 2002) was used in order to visualize the interactions. 7 weak polar conctacts (orange), 3 polar conctact (red), 2 hydrogen bond (cyan), 3 weak hydrogen bonds (green), 21 hydrophobic contact (magnetta) were found for 5NLX/ZEA. For 5NLX/CFF complex, 5 weak polar contact (orange), 1 polar contact (red), 1 hydrogen bond (cyan), 2 weak hydrogen bonds (green) were found. The other interactions that not mentioned here include aromatic contacts, carbonyl interactions or other type of interactions. **B.** Both 5NLX/ZEA and 5NLX/CFF complex were placed into membrane via membrane protein tutorial (Aksimentiev et al., 2009). Zeatin: ZEA, Caffeine: CFF

Based on crystal structure of A2AR (PDB entry:5MZP), caffeine was previously reported to interact with residues ILE 66, VAL 84, PHE 168, GLU 169, MET 177, LEU 249, ASN 253, MET 270 and ILE 274 (Carpenter et al., 2017; Cheng et al., 2017). The residues of the structure of A2AR (PDB Entry:5MZP) was renumbered be able to compare with the structure of A2AR (PDB Entry:5NLX) used in our model. The corresponding residues are detected as ILE 75, VAL 93, PHE 177, GLU 178, MET 186, LEU 354, ASN 358, MET 375 and ILE 379, respectively. As the result of docking of ligands to receptor, it was determined that caffeine interacts with residues ILE 379, ASN 358, LEU 354, MET 186, VAL 93 while zeatin interacts with residues MET 186, PHE 177, VAL 93, ALA 90, ILE 75, ALA 72, ILE 379, LEU 354, ASN 358 based on our model. This finding showed that zeatin have ability to form more interaction than caffeine in the same binding pocket. 181 contacts in total were detected for 5NLX/ZEA complex while 156 contacts were detected for 5NLX/CFF complex. 7 weak polar conctacts, 3 polar conctact, 2 hydrogen bond, 3 weak hydrogen bonds, 21 hydrophobic contacts were found for 5NLX/ZEA. The hydrogen bonds were formed with the aminoacid residues ASN 358, ALA 90 and ALA 72; polar contacts were formed with ALA 90, ILE 379, ILE 75 and VAL 93 and hydrophobic contacts were formed with MET 186, PHE 177 and LEU 354. For 5NLX/CFF complex, 5 weak polar contact, 1 polar contact, 1 hydrogen bond, 2 weak hydrogen bonds were found. The hydrogen bond were formed with MET 186, ASN 358 and LEU 354; polar contacts were formed ILE 379 and VAL 93. As A2AR is a transmembrane protein, both 5NLX/CFF and 5NLX/ZEA complexes were aligned into membrane (Figure 1B) for the MD simulations.

The hydrophobic pocket residues of A2AR were simulated for 50 ns to compare the adjacency of zeatin and caffeine. RMSD of protein backbone atoms of 5NLX/ZEA and 5NLX/CFF complexes for 3 production runs reached to a steady plateau after 10 ns, indicating stable simulations for analysis (Figure 2A). To explore whether if caffeine and zeatin were held in close proximity to the hydrophobic pockets, the fluctuations of the atoms of caffeine and zeatin were analyzed and the average of 3 production runs was taken for both 5NLX/ZEA and 5NLX/CFF complex. Zeatin showed lower flexibility than caffeine with A2AR based on RMSF as an expected result (Figure 2C) because zeatin has the ability to form more interaction than caffeine, which reduces the flexibility of zeatin.

The interactions between ligands (zeatin and caffeine) and A2AR are given (Table 1) during 50 ns MD simulation. While hydrogen bonds between A2AR and zeatin were mostly formed with the residues MET 375 and HIS 383, hydrogen bonds between A2AR and caffeine were mostly

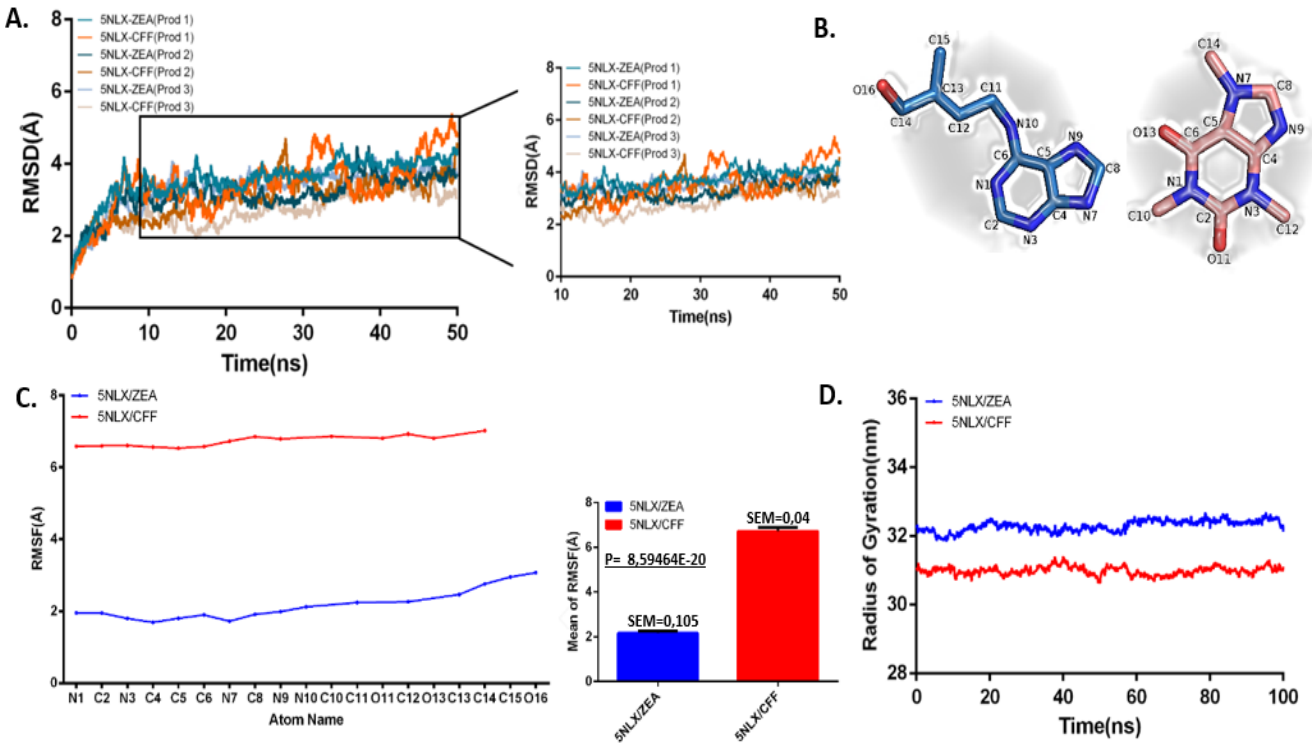

Figure 2: *Identification of 5NLX/ZEA and 5NLX/CFF complexes during MD simulations.*

A. RMSD of backbone atoms of 5NLX/ZEA and 5NLX/CFF complexes was plotted for 3 production run. RMSD of backbone atoms for both complex and for each run was calculated in 3 Å in order to detect the similarity between structures and production runs. The required stability was reached after 10 ns. **B.** The atoms of ligands (zeatin and caffeine) were described in the image. **C.** RMSF of the atoms of zeatin for 5NLX/ZEA (red) complex versus RMSF of the atoms of caffeine for 5NLX/CFF (blue) complex were analyzed in order to compare two structure in 50 ns MD simulation. It is determined that zeatin (blue) showed lower flexibility than caffeine (red). **D.** Radius of gyration which means the distrubition of molecules of all compound was measured for both 5NLX/ZEA (red) and 5NLX/CFF (blue) complex and it showed that structure shape stay stable during 50 ns MD simulation. Å: Angstrom, C: Carbon, O: Oxygen, N: Nitrogen

formed with residues ASN 358. Furthermore, the number of hydrogen bonds on frames are also given (Figure 3). Hydrophobic interactions between A2AR and zeatin were mostly formed with the residues ILE 379, LEU 354, ALA 382, PHE 177, TYR 376, HIS 383, ILE 75 while hydrophobic interctions between A2AR and caffeine were mostly formed with the residues PHE 177, GLU 178, ILE 379, HIS 369. Salt bridges were only observed between A2AR and caffeine with the residues GLU 178. These residues were detected based on higher occupancy (>50%) between 999 frames during 50 ns MD simulations. It was determined that most of the residues interact with both zeatin and caffeine but the occupancy between the frames is higher for zeatin and the interactions with the occupancy under 50% for both zeatin and caffeine were not analyzed in this study. For instance, Interaction between zeatin and ILE 379 was observed with the percentage of 86.4% between frames for 5NLX/ZEA çomplex while it was observed with the percentage of 62.8% for 5NLX/CFF complex. Salt bridges were not detected for 5NLX/ZEA complex during the analysis. These findings indicates that zeatin and caffeine display common residue interactions the same binding pocket.

Table 1: The summary of interactions between A2AR with ligands (zeatin and caffeine).

5NLX/ZEA				
Interactions after docking			Interactions during Simulation	
Hydrogen bonds	Polar Bonds	Hydrophobic Bonds	Hydrogen Bonds	Hydrophobic Bonds
ALA 72	ALA 90	MET 186	MET 375	ILE 379
ASN 358	ILE 75	LEU 354	HIS 383	LEU 354
ALA 90	VAL 93	PHE 177		ALA 382
	ILE 379			PHE 177
				TYR 376
				HIS 383
				ILE 75

5NLX/CFF				
Interactions after docking		Interactions during Simulation		
Hydrogen bonds	Polar Bonds	Hydrogen Bonds	Hydrophobic Bonds	Salt Bridges
LEU 354	ILE 379	ASN 358	PHE 177	GLU 178
MET 186	VAL 93		GLU 178	
ASN 358			ILE 379	
			HIS 369	

After the docking of both ligands (zeatin and caffeine) to A2AR (PDB entry:5NLX), interacting residues were observed via Arpeggio (Jubb et al., 2017) as in the table. For MD simulations, interacting residues were detecting between the frames by using VMD Humphrey et al. (1996) and the residues that have higher occupancy (>50%) between 999 frames were choosen during the analysis of hydrogen bonds,hydrophobic bonds and salt bridges.

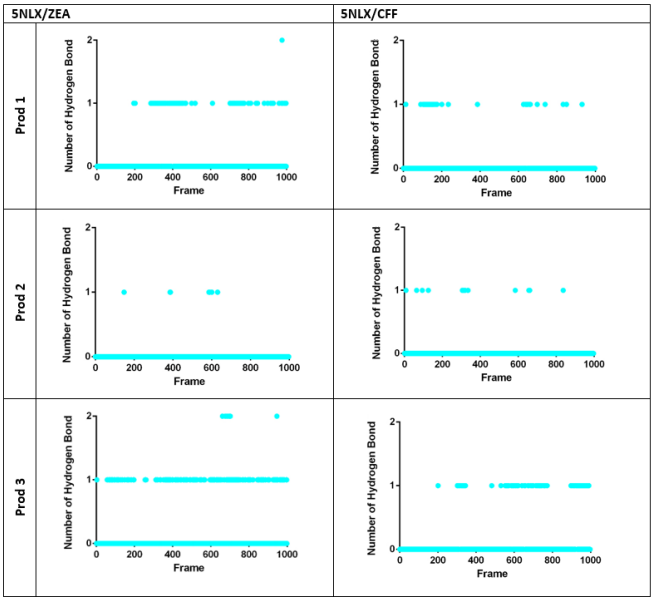

Figure 3: The plots of hydrogen bonds for 5NLX/ZEA and 5NLX/CFF complex.

Hydrogen bond anlaysis was performed in VMD Humphrey et al. (1996) for 3 productions run based on frames in 3 Å. 5NLX/ZEA complex contains more hydrogen bonds than 5NLX/CFF complex for each production run in 50 ns MD simulation.

After the detection of critical residues for both 5NLX/ZEA and 5NLX/CFF complex, distance for 50 ns and RMSF for 20-50 ns were analyzed based on the critical residues. In distance analysis, zeatin stayed stable with the residues TYR 376 (Supplementary Figure 1), LEU 354 (Supplementary Figure 2), ILE 379 (Supplementary Figure 3), MET 375 (Figure 4), PHE 177 (Figure 5), ALA 382 (Figure 7), ILE 75 (Supplementary Figure 4), HIS 383 (Supplementary Figure 6) during 50 ns while caffeine is fluctuated after 20 ns. Caffeine and zeatin get closer during interaction with the residue GLU 178 (Figure 6) between 20-50 ns. For the residue ASN 358 (Supplementary Figure 5), zeatin and caffeine get closer between 20-30 ns and then caffeine moves away from the residue ASN 358. In addition, caffeine and zeatin get closer to HIS 369 (Supplementary Figure 7) between 30-50 ns. Therefore, It was expected that RMSF of this residues would be lower for 5NLX/ZEA complex than 5NLX/CFF complex because zeatin stays more stable during the interaction with these residues in 50 ns MD simulation. RMSF values based on each reasidue was calculated between 20-50 ns but RMSF of these residues were found as similar for both 5NLX/ZEA and 5NLX/CFF complex in our model.

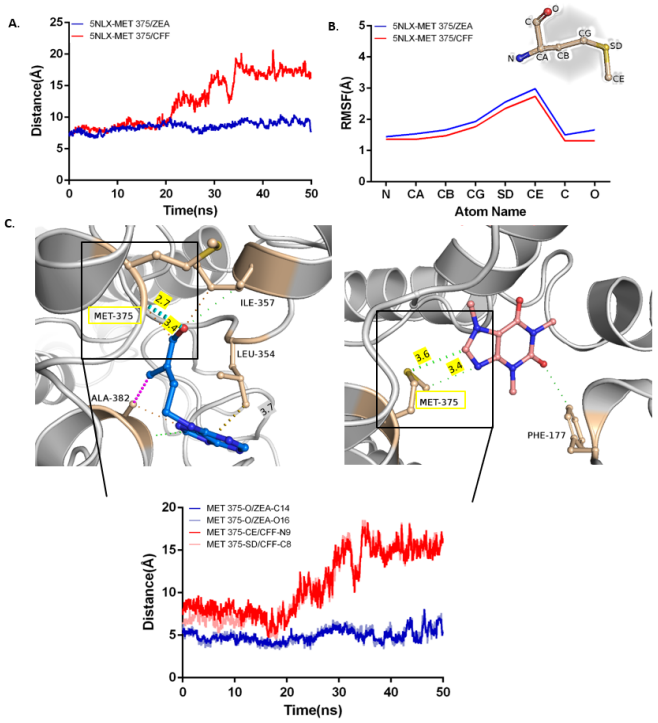

Figure 4: The comparison of interactions with the residue MET 375 for 5NLX/ZEA and 5NLX/CFF complex via analysis of distance and RMSF.

A. Distance of zeatin (red) and caffeine (blue) to the residues MET 375 of A2AR receptor were measured via VMD (Humphrey et al., 1996) . While zeatin stays stable in ~5 Å distance during 50 ns MD simulation, caffeine moves away after 20 ns, from ~5 Å to ~15-20 Å. **B.** Based on RMSF of backbone atoms of the residue MET 375 for both 5NLX/ZEA (red) and 5NLX/CFF (blue) complexes, the flexibility of the residue MET 375 was observed as similar for both 5NLX/CFF and 5NLX/ZEA complexes. Distance analysis provide better understanding for the comparison of the effect of caffeine and zeatin on A2AR, shows that zeatin stays longer with the residues caffeine interacts normally. **C.** The image that was obtained from 422. frame (21,1. Ns) of first production run for 5NLX/ZEA and 327. frame (16,35. Ns) for 5NLX/CFF specifically show the interaction with the residue MET 375 and zeatin. The interaction was highlighted as polar interaction (orange) with the distance 3.4 Å and hydrogen bond (cyan) with the distance 2.7 Å for 5NLX/ZEA, weak hydrogen bonds (green) with distance 3.6 and 3.4 Å for 5NLX/CFF via PyMol (Himer et al., 2010). Distance was also calculated in order to observe the interaction in all 50 ns MD simulation as atom-atom. It was determined from selected images that the interaction performs between the O atoms of residue MET 375 and the C17, O16 atoms of zeatin in one hand while the interaction performs between the CE, SD atom of residue MET 375 and the C8, N9 atoms of caffeine in other hand. The atom-atom distance graph showed that interaction stays stable in 5 Å distance for 5NLX/ZEA complex but distance was increasing after 20 ns for 5NLX/CFF complex. This result indicates that when zea interacts with the residue MET 375, CFF moves away from the interaction point. Zeatin: ZEA, Caffeine: CFF, Å: Angstrom, C: Carbon, CA (Cα): alpha carbon, CB (Cβ): Beta carbon, CG (Cγ): Gamma carbon, CE (Cε): Epsilon carbon O: Oxygen, SD (Sδ): Delta sülfür, N: Nitrogen

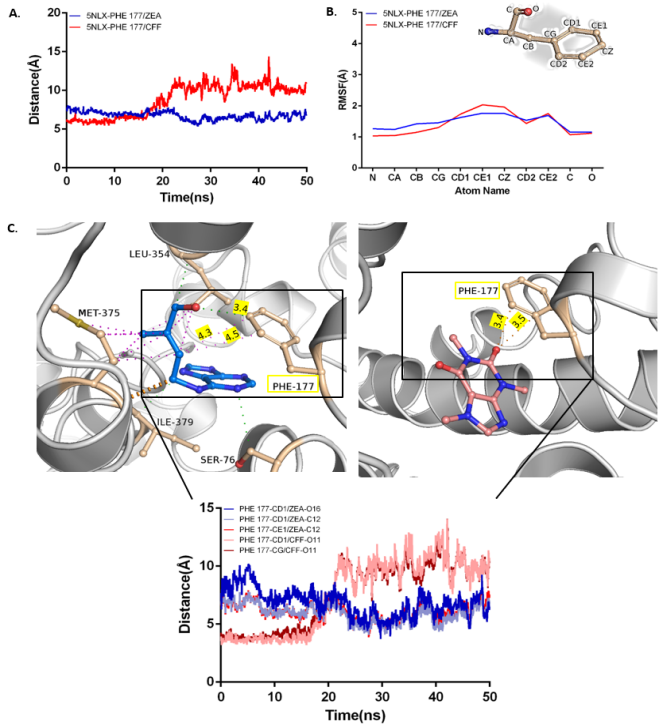

Figure 5: The comparison of interactions with the residue PHE 177 for 5NLX/ZEA and 5NLX/CFF complex via analysis of distance and RMSF.

A. Distance of zeatin (red) and caffeine (blue) to the residues PHE 177 of A2AR were measured via VMD (Humphrey et al., 1996). While zeatin stays stable in ~5-10 Å distance during 50 ns MD simulation, caffeine moves away after 20 ns FROM 5 Å to ~10-15 Å. **B.** Based on RMSF of backbone atoms of the residue PHE 177 for both 5NLX/ZEA (red) and 5NLX/CFF (blue) complexes, the flexibility of the residue PHE 177 was observed as almost similar for both 5NLX/CFF and 5NLX/ZEA complex. Distance analysis provide better understanding for the comparison of the effect of caffeine and zeatin on A2AR, shows that zeatin stays longer with the residues caffeine interacts normally. **C.** The image that was obtained from 554. frame (27.7. ns) of first production run for 5NLX/ZEA and 544. frame (27,2. Ns) for 5NLX/CFF specifically shows the interaction with the residue PHE 177 and zeatin. The interaction was highlighted as hydrophobic interaction (purple) with the distance 4.3 and 4.5 Å and weak hydrogen bond (green) with the distance 3.4 Å for 5NLX/ZEA, polar contacts (orange) with distance 3.4 and 3.5 Å for 5NLX/CFF via PyMol (Himer et al., 2010). Distance was also calculated in order to observe the interaction in all 50 ns MD simulation as atom-atom. It was determined from selected images that the interaction performs between the CD1, CE2 atoms of residue PHE 177 and the C12, O16 atoms of zeatin in one hand while the interaction performs between the CD1, CG atom of residue PHE 177 and the O11 atoms of caffeine in other hand. The atom-atom distance graph showed that interaction stays stable in 5-10 Å for 5NLX/ZEA complex but distance was increasing after 20 ns for 5NLX/CFF complex. This result indicates that when zeatin interacts with the residue, caffeine moves away from the interaction point. Zeatin: ZEA, Caffeine: CFF, Å: Angstrom, C: Carbon, CA (Cα): alpha carbon, CB (Cβ): Beta carbon, CG (Cγ): Gamma carbon, CD (Cδ): Delta carbon, CE (Cε): Epsilon carbon, CZ (Cζ): Zeta carbon, O: Oxygen, N: Nitrogen

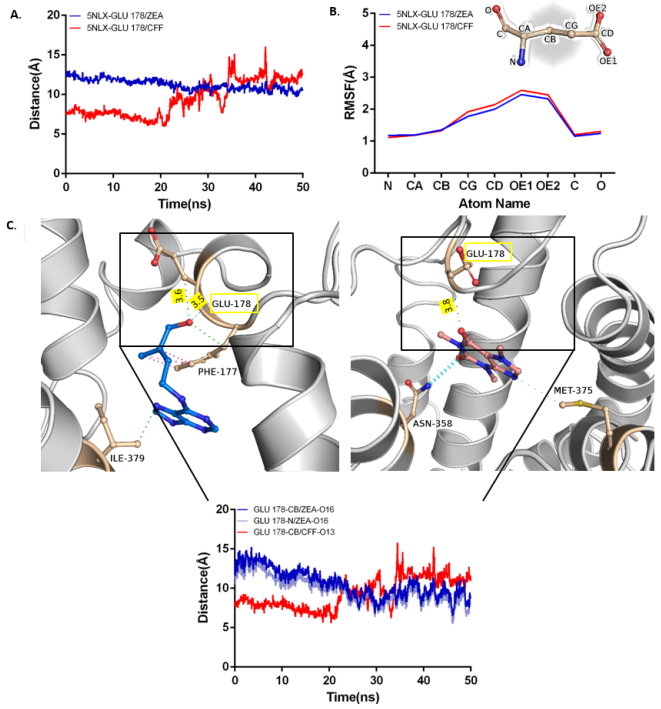

Figure 6: The comparison of interactions with the residue GLU 178 for 5NLX/ZEA and 5NLX/CFF complex via analysis of distance and RMSF.

A. Distance of zeatin (red) and caffeine (blue) to the residues GLU 178 of A2AR measured via VMD (Humphrey et al., 1996). While zeatin stays stable in ~10-15 Å distance during 50 ns MD simulation, caffeine stays stable until 20 ns then, it gets closer with GLU 178 between 20-50 ns, reaching ~15 Å. This data also indicates that caffeine and zeatin stays close position to each others after 20 ns. **B.** Based on RMSF of backbone atoms of the residue GLU 178, both 5NLX/ZEA (red) and 5NLX/CFF (blue) complexes showed that flexibility of the residue GLU 178 was observed as similar for both 5NLX/CFF and 5NLX/ZEA complexes. The possible interaction was formed with another residue can reduce the the flexibility of GLU 178 for 5NLX/CFF complex **C.** The image that was obtained from 924. frame (46,2. ns) of first production run for 5NLX/ZEA and 707. frame (35,35. ns) for 5NLX/CFF specifically show the interaction with the residue GLU 178 and ZEA. The interaction was highlighted as weak hydrogen bond (green) with the distance 3.6 and 3.5 Å for 5NLX/ZEA and 3.8 Å for 5NLX/CFF via PyMol (Himer et al., 2010). Distance was also calculated in order to observe the interaction in all 50 ns MD simulation as atom-atom. It was determined from selected images that the interaction performs between the CB, N atoms of residue E178 and the O16 atom of zeatin in one hand while the interaction performs between the CB atom of residue GLU 178 and the O13 atom of caffeine in other hand. The atom-atom distance graph showed that interaction stays stable in 10-15 Å for 5NLX/ZEA complex but distance was increasing after 20 ns for 5NLX/CFF complex and caffeine gets closer position with the residue and zeatin. This result indicates differently from other residues that both zeatin and caffeine stay stable with the residue GLU 178 after 20 ns which supports the previous RMSF results. GLU 178 can play critical role in stability of caffeine as a salt bridge. Zeatin: ZEA, Caffeine: CFF, Å: Angstrom, C: Carbon, CA (Cα): alpha carbon, CB (Cβ): Beta carbon, CG (Cγ): Gamma carbon, CD (Cδ): Delta carbon, O: Oxygen, OE (Oε): Epsilon oxygen, N: Nitrogen

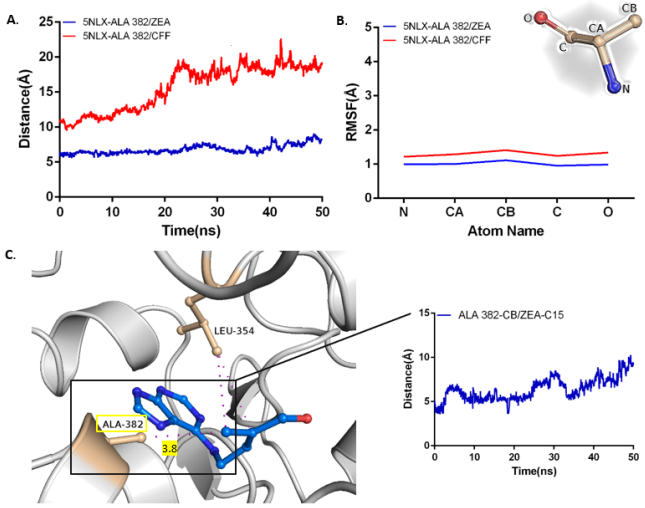

Figure 7: The comparison of interactions with the residue ALA 382 for 5NLX/ZEA and 5NLX/CFF complex via analysis of distance and RMSF.

A. Distance of zatin (red) and caffeine (blue) to the residues ALA 382 of A2AR were measured via VMD (Aksimentiev et al., 2009). While zeatin stays stable in ~5-10 Å distance during 50 ns MD simulation, caffeine moves away after 20 ns from 10 Å to ~20 Å. **B.** Based on RMSF of backbone atoms of the residue ALA 382, both 5NLX/ZEA (red) and 5NLX/CFF (blue) complexes showed that flexibility of the residue ALA 382 is lower for 5NLX/ZEA as expected and this residue may play important role the stability of zeatin during the interaction with A2AR. **C.** The image that was obtained from 457. frame (22,85. ns) of first production run for 5NLX/ZEA specifically shows the interaction with the residue ALA 382 and zeatin. The interaction was highlighted as hydrophobic interaction (purple) with the distance 3.8 Å for 5NLX/ZEA via PyMol (Himer et al., 2010). Distance was also calculated in order to observe the interaction in all 50 ns MD simulation as atom-atom. It was determined from selected images that the interaction performs between the CB atom of residue ALA 382 and the C15 atom of zeatin. No interaction was detected between caffeine and the residue ALA 382 during analysis with the occupancy >50%. The atom-atom distance graph showed that interaction stays stable in 5 Å until ~25 ns and after, distance reachs 10 Å. Zeatin: ZEA, Caffeine: CFF, Å: Angstrom, C: Carbon, CA (Cα): alpha carbon, CB (Cβ): Beta carbon, O: Oxygen, N: Nitrogen

The movement of ligands (zeatin and caffeine) was analysed in each production run and it was detected that caffeine moves outward direction while zeatin moves inward direction during 50 ns MD simulation (Figure 8). The reason of this movement may be the formed new interactions with other residues in 5NLX/CFF complex during the simulation, leading the reduction of flexibility of the residues but these all findings proof that zeatin has ability to stay more stable during interaction with A2AR and interacts with either the same or different residues in order to stay longer in the binding pocket compared to caffeine. Zeatin may have potential role as ligand on A2AR and promising a new target for further studies.

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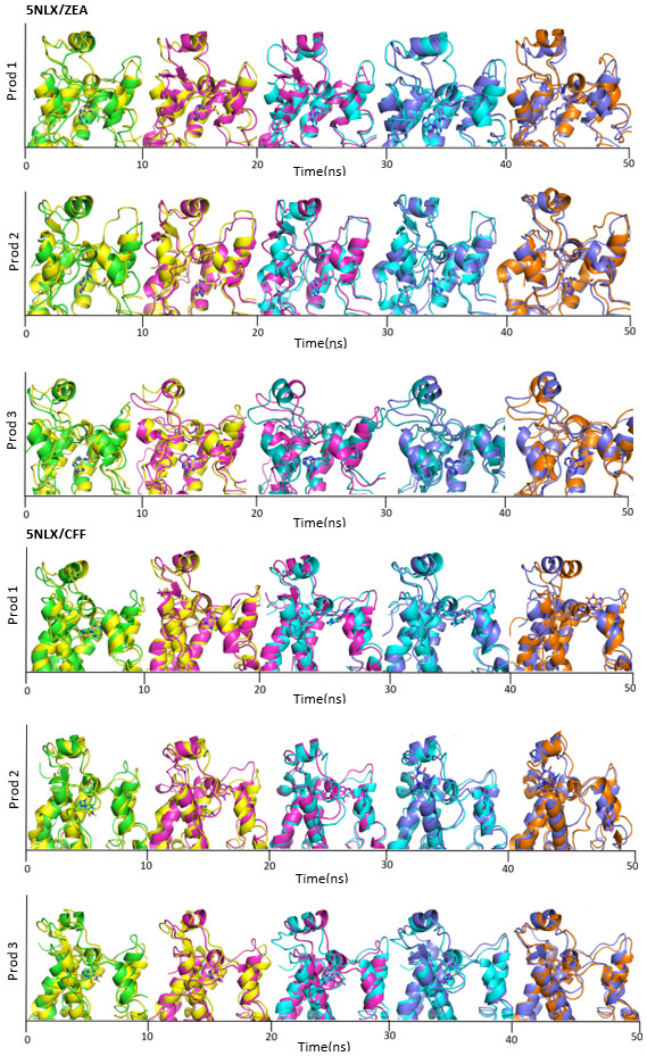

Figure 8: The movement of ligands (zeatin and caffeine) complex within 50 ns MD simulation for 3 production runs.

In 5NLX/ZEA complex, zeatin moves inward direction while caffeine moved outward direction in 5NLX/CFF complex during 50 ns and the most possible reason for the movement of caffeine is the formed new interactions between the different residues of protein and caffeine. Each color represents the structure in certain time. Structure in 0th ns: Green, Structure in 10th ns: Yellow, Structure in 20th ns: pink, Structure in 30th ns: cyan, Structure in 40th ns: purple, Structure in 50th ns: Orange

4. Discussion

A2ARs play a key role in regulating transmembrane signaling pathways in response to specific ligand and caffeine is known as one of the nonselective A2AR antagonist as well as is the most consumed psychostimulant in the World (Stevens et al., 2013; Boia et al., 2016). The main action of caffeine involves the blockade of A2AR on a various of physiological process.[35] Despite of harmful effect of caffeine when it is overtaken, the main reason for consumption of caffeine is to in cognitive performance and mood (Smith et al., 2013; Pasman et al., 2017). However, it enhances performance more in fatigued than well-rested subjects (Lorist et al., 1994; Childs et al., 2008). It has recently been demonstrated that zeatin which is one of the plant hormones activate the mammalian A2AR, playing a role in the regulation of cells involved in both innate and adaptive immunity as well as protect cognitive dysfunction such as improves memory impairment or mood disorders (Lappas et al., 2015; Kim et al., 2008). The interaction mechanism of zeatin on A2AR has not been demonstrated clearly yet. The binding of ligand on A2AR causes the conformational changes on the receptor and leads the activation of signalling pathways (Carpenter et al., 2017; Dalton et al., 2015). The model structure of A2AR in complex with zeatin and caffeine (as positive control) were constructed for this study and analyzed within 50 ns MD simulation with the repeated 3 production runs. Because of the size of system, the simulation time was kept as 50 ns for each production run but it can be extended for future studies to provide better estimation. The results indicate that both zeatin and caffeine have interaction in the same hydrophobic pocket. The residues TYR 376, LEU 354, ILE 379, MET 375, PHE 177, ALA 382, ILE 75, ASN 358, GLU178, HIS 383, HIS 369 were determined as critical for the interaction between A2AR and both ligands. Residue-specific distance showed that zeatin stays more stable than caffeine during 50 ns MD simulation, supporting zeatin as a new target. Despite of the distance analysis, RMSF of each residue showed close results for both 5NLX/ZEA and 5NLX/CFF complex. This finding provides a new question for the further studies, how the flexibility of the protein residues in 5NLX/CFF complex based on residue-specific RMSF analysis can be observed almost the same with in 5NLX/ZEA complex although zeatin is determined as more stable based on distance analysis? In addition to, zeatin was also determined as less flexible during RMSF analysis for ligands (Figure 2). The most possible reason for it can be because of new performed interactions occuring on the specific residue with other residues of protein during 50 ns MD simulation. As the residues are allowed to interact for a period of time to observe dynamic evolution of the system, the flexibility of the specific residue can be observed as less for both models. The combination of study with distance analysis provided more accurate and sensitive results to detect the stability of zeatin on A2AR. Although, zeatin was detected as stable in most of the choosen residues, the residue ALA 382 may plays important role in stabilization of zeatin in complex because the interaction with the residue ALA 382 only was performed in 5NLX/ZEA complex and the flexibility of the residue was lower for 5NLX/ZEA complex, indicating longer interaction during 50 ns MD simulation. During the study, local rigidity was considered to understand the stability of binding by looking RMSD, RMSF and distance analysis but these considerations were not considered enough to understand exactly stability of binding and binding affinity. For further studies, binding energy estimation is required to detect the effect of zeatin on A2AR and the strength of the binding interaction. By this study, zeatin is identified as potential ligand of A2AR based on computational model for the first time and the interacting resdiues was analysed in order to provide better understanding of the binding mechanism based

on positive control described as A2AR in complex with caffeine.

5. Conclusion

Zeatin, a plant hormone, has recently been detected as potential target for A2AR but protein-ligand interaction mechanism has not been clarified yet. Based on structural modelling, zeatin was identified as a ligand of A2AR for the first time and interacting resdiues were analyzed based on A2AR in complex with caffeine as positive control to reveal binding mechanism.

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