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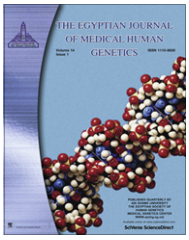

ORIGINAL ARTICLE

Computational evaluation of small molecule inhibitors of RGS4 to regulate the dopaminergic control of striatal LTD

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Abstract Parkinson's disease is a neurodegenerative disease which is the result of the degradation of the dopaminergic neurons in the substantia nigra pars compacta, leading to a disregulation of thalamocortical circuits. Traditional treatment involves the use of levodopa which increases the dopamine level in the striatum. There is a need for alternative non-dopamine therapy to prevent the side effects of the conventional drugs used. Recently small molecule inhibitors of RGS have become the prime candidates in studies related to regulating RGS by binding to its allosteric site and thus changing its structure. Through the docking studies we observed that these small molecule modulators of RGS4 make stable complexes with RGS4 when compared to native RGS4. The Gq(alpha)–RGS4–drug complexes are less stable. The increase in flexibility of the RGS4–drug complex could be the reason for the inability of the RGS4–drug complex to bind to the G protein. In

Abbreviations: RGS, Regulator of G protein; SNpc, Substantia Nigra pars compacta; mGluR, metabotropic glutamate receptors; MSNs, Mini Spinny Neurons; LTD, Long Term Depression; eCB, endocannabinoid; AMP, Adenosine Mono Phosphate; PKA, phosphorykinase A; L-VGCCs, L-type voltage-gated calcium channels; AEA, anandamide; PLD, phospholipase D; PDB, Protein Data Bank; 2-AG, 2-arachidonoylglycerol; HADDOCK, High Ambiguity Driven protein-protein DOCKing; CPORT, Consensus Prediction Of interface Residues in Transient complexes; CPORT, Consensus Prediction Of interface Residues in Transient complexes; SMPPIIs, small molecule protein-protein interaction inhibitor; GTP, Guanosine Triphosphate; SMILES, Simplified molecular input line entry system

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our docking results, CCG63802 formed the most promising drug as a RGS4 inhibitor as it formed the most stable complex with RGS4 and also formed the least stable complex, Gq(alpha)–RGS4–CCG63802 complex. In our studies we evaluated the therapeutic potential of the small molecule inhibitors to provide a prospective treatment for Parkinson's disease.

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1. Introduction

Parkinson disease is a common nervous system disorder associated with ageing. This is the result of the degeneration of dopamine synthesizing neurons in the substantia nigra pars compacta (SNpc) that innervate the striatum. The dopamine in the brain is produced by the dopaminergic neurons. These neurons are irreproducible therefore once lost cause a great loss to the human brain. The main symptoms of the disease are tremors, problems with balance and walking, jerky stiff movements, bradykinesia and rigidity.

The basal ganglia are a network of subcortical brain nuclei engaged in many aspects of motor function, this includes action, selection and adaptive motor learning [1–4]. Striatal projection neurons(MSNs) regulates the motor functions through the plasticity of excitatory synapses .The molecular mechanisms underlying striatal plasticity which sets the gain on signals driving both direct and indirect-pathway basal ganglia circuits is still not completely understood. The best-studied form of striatal plasticity is endocannabinoid-dependent Long Term Depression (eCB-LTD). This form of Long Term Depression is induced following the production and release of endocannabinoids (eCBs) from the postsynaptic neuron, which then acts on presynaptic CB1 receptors to lower neurotransmitter release probability. Both direct and indirect pathways are regulated by the D2 and A2A receptors acting through cAMP/PKA, which further regulates the production of the endocannabinoids through mGluR [5].

The effect of the loss of dopamine receptors in the striatum is still unknown, how it controls the function is especially important in the context of Parkinson's disease. The indirect-pathway is more reliably reproduced in vitro therefore it is widely used to express the working of the various proteins involved in eCB-Ltd. The postsynaptic membrane proteins that are required to elicit eCB release sufficient to induce indirect-pathway eCB-LTD: group I (Gq-coupled) metabotropic glutamate receptors (mGluRs), L-type voltage-gated calcium channels (L-VGCCs), and dopamine D2 receptors [6–10]. Adenosine A2A receptors may modulate indirect-pathway LTD also [11,12]. The actual process of the mobilization of eCBs was still obscure until two main candidates for the eCBs were found (1) anandamide (AEA), thought to be produced by phospholipase D (PLD) activity, and (2) 2-arachidonoylglycerol (2-AG), thought to be produced by PLCb and DAG lipase [13,14]. Much of the available studies have supported the role of AEA in indirect-pathway LTD [15–17].

It remains unclear why the activation of D2 receptors is required for eCB-LTD and how the blockade of A2A receptors enhances it. In a study conducted to understand dopamine (DA)-dependent corticostriatal plasticity showed that D2 receptors act via adenylyl cyclase 5 [18], it was also seen in another study which utilized HFS-LTD, that the D2 receptors also promotes the eCB-LTD through the reduction in cAMP levels or PKA activation. It was concluded that increased cAMP/PKA activity inhibits LTD[5]. It was understood that as both D2 and A2A receptors, both Gs regulated, regulate the eCB production therefore they must be acting on a common target. Through studies it was understood that the group I mGluR and Gq form the primary candidates for sites which can be used to manipulate this cellular process. The role of mGluR was clarified and it was found that the inhibition of mGluR-Gq signalling prevents the mobilization of both 2-AG and AEA [5], thus inhibiting the eCB-Ltd.

The Regulator of G protein (RGS) is a group of protein that regulates the life time of the active G alpha-GTP complex by accelerating the GTP hydrolysis. RGS4 is expressed strongly in MSNs in the dorsolateral striatum, where it regulates the activity of mGluR5 and PLCb [19,20], its activity is increased by PKA phosphorylation [21], and it strongly inhibits its signalling through Gq [22]. RGS4 is expressed in both direct-pathway MSNs and cholinergic interneurons [23] its loss may also be contributing to the effects of dopamine depletion [24]. We only consider the effect of RGS4 in the postsynaptic neuron as it was observed that RGS4 protein production is manipulated by the cAMP/PKA which itself is modulated by the D2 and A2A receptors [5].

Dopamine depletion has profound effects on the expression of RGS proteins in the striatum, in particular RGS4 [25,26]. The D2 receptor negatively regulates cAMP/PKA while A2A positively regulates it. Dopamine provides a damping effect; it makes sure that muscles work smoothly, under precise control, and without unwanted movement. Another transmitter, acetylcholine, inhibits the damping effect. Parkinson's disease is a result when the effect of dopamine is less than that of acetylcholine. Dopamine deficiency rather than acetylcholine excess is normally responsible for this occurring. In addition, Mono Amine Oxidase-B breaks down the excess dopamine in the synapse further diminishing the dopamine that is left in the substantia nigra [27].

Most drug treatments increase the level of dopamine in the brain or oppose the action of acetylcholine. Levodopa which is a precursor for dopamine is widely used to compensate for the dopamine loss in Parkinsonian patients. The drug is useful for the initial stages of the disease but as the disease progresses the drug becomes less effective. The patient may also experience some side effects such as increase in involuntary actions and dyskinesia which is one of the main problems. Therefore there is a need for dopamine independent drugs. The reduced behavioural deficits following dopamine depletion in RGS4 deficient mice indicate that RGS4 inhibition may be an effective non-dopamine dependent strategy for treating Parkinson's disease.

Many small peptide inhibitors of RGS4 and its related family members [28,29] are reported. These peptides have a sequence similar to the switch1 and switch 2 regions of the RGS4 and bind to the G protein's A site .The peptide inhibitors are not preferred because of the physical properties of the

peptides, they function in a cellular environment only when they are administered intracellularly [e.g., by dialysis via a patch pipette [28]. Genetic studies would bolster research in drug discovery [30–33]. Research in small molecules' protein–protein inhibitors consequently identified novel RGS inhibitors that retain activity under reducing conditions and ones that have a reversible mechanism of action [34]. These compounds, CCG63808 and CCG63802 bind to a B site, allosteric site where it causes a destabilizing of the RGS4 protein. CCG4968 also inhibits RGS4 but it binds more strongly to the cysteines in the RGS and therefore forms an irreversible bound state [35].

In our study we evaluated the therapeutic potential of the three drugs which are the RGS inhibitors. The main purpose was to explore the possibility of a non-dopamine therapy for Parkinson's disease. The drugs were docked with RGS4 to find their binding energy needed and the stability of the complexes. It was then observed that when the RGS4–drug complex was bound with Gq then the binding energies were lower than the binding energy needed for the native Gq–RGS4 complex, thus proving that these complexes were less favourable than the native complex. The RGS4–drug complexes are less porous than the native RGS4 protein. Conformational flexibility of a protein molecule affects its interaction with the ligand and its biological partners at different levels [36–43]. The distance fluctuation between two C alpha atoms was studied to observe the flexibility of the complexes around the interactive residues. It was also observed that the RGS4–drug complexes are more flexible than the native RGS4 in its bound state with Gq, this structural change could be the reason because of which the RGS4–drug complex is inhibited from attaching to Gq. Thus it was concluded that these small drugs which are inhibitors of RGS4, regulate the eCB-LTD and therefore can potentially be used to treat Parkinson's disease.

2. Materials and methods

2.1. Dataset

The two sequences for the purposes of this study were taken from PDB (Protein Data Bank): 3AH8 (Gq) [44] and 1AGR (Galphai1–RGS4) [45]. The structures of the 3 drug molecules that we used for our research were obtained from their literatures, CCG4986 [46], CCG63802 and CCG63808 [34]. Three dimensional molecular structures for drug molecules were derived from CORINA web server. SMILE notation of the drug molecules was given as input to the server which was used to generate the PDB file of the 3D structure.

2.2. Protein–ligand docking

Protein–ligand interactions were calculated by using Autodock 4.2 [47]. Autodock combines an empirical free energy force field with a Lamarckian Genetic Algorithm, providing fast prediction of bound conformations with predicted free energies of association. The primary method for conformational searching is a Lamarckian genetic algorithm in which a population of trial conformations is created, and then in successive generations these individuals mutate, exchange conformational parameters, and compete in a manner analogous to biological evolution, ultimately selecting individuals with lowest binding energy. PDB files of the complex thus formed with the lowest energy were considered.

2.3. Protein–protein interactions

Protein–protein interactions play a pivotal role in various aspects of the structural and functional organization of the cell and their elucidation is crucial for a better understanding of processes such as metabolic control, signal transduction, and gene regulation. Protein–protein docking was done using HADDOCK web server [48]. HADDOCK (High Ambiguity Driven protein–protein DOCKing) encodes information from the identified or predicted proteins in ambiguous interaction residues to drive the docking process. Gq protein and the RGS4–ligand complex were uploaded in the web server to obtain docked complexes.

2.4. Prediction of functional sites

InterProSurf was used to predict interacting functional amino acid on a protein surface. The prediction method is based on solvent accessible surface area of residues in the isolated subunits, a propensity scale for interface residues and a clustering algorithm to identify surface regions with residues of high interface propensities [49]. Amino acids whose change in accessible surface area was above 45.0 were chosen as the interacting residues in our study. The PDB files of the protein were uploaded to be evaluated.

CPORT (Consensus Prediction Of interface Residues in Transient complexes) was also used to obtain the interacting residues on the surface of the given complexes. The server combines six interface prediction web servers to give a consensus method [50]. The Gq–RGS4–drug complexes were given as an input to the server.

2.5. Protein movement analysis

ElNémo the Elastic Network Model [51] was used to find out the neighbouring residues of the amino acids chosen by InterProSurf and CPORT, to check their flexibility. The present version of elNémo allowed us to compute the low frequency normal modes for a given protein structure in PDB format. We were able to analyse these modes at different levels of detail, i.e. compare the collectivity of the modes, view 3-D animations of the protein movement for each mode and identify those residues that have the largest distance fluctuations in a given mode. The map for distance fluctuations between residues i and j measures the relative moments between residues in the mode k which is also provided by the server. In such maps, rigid and flexible blocks of amino acid residues can be identified, as well as their relative moment can be studied. The blocks of amino acid residues which behave as rigid bodies during the motion appear in white in the map, whereas flexible segments are filled with dark blue or light blue colour. Dark blue colour indicates that the distance between two C alpha atoms increases significantly, and a light blue symbols that it decreases. Every pixel corresponds to a single residue. Grey lines are drawn every 10 residues, blue lines every 100 residues. We gave the Gq–RGS4–drug docked complexes as input to elNémo.

3. Result and discussion

RGS4, a GTPase was identified as the regulator for the Gq-protein by accelerating its inactivity. This GTPases are abundantly found in the striatum, the part of the brain that controls movement. In models of Parkinson's disease in mice, the researchers at the Gladstone Institute found that RGS4 actually contributes to problems with motor control, leading to a deterioration of movement and motor coordination [5]. In our studies, we evaluated the effect of three drugs which act as inhibitors for protein–protein interaction between RGS4 and Gq(alpha). Inhibition of signalling networks through the disruption of protein–protein interactions presents unique new targets for the development of chemical tools and for possible therapeutic drug discovery [52,53]. The small molecules inhibit the downstream process of the mGluR which is the main target for the regulation of the endocannabinoid-dependent Long Term Depression (eCB-LTD). The traditional drugs for example; levodopa, used in the treatment increase the dopamine level but as the disease progresses it is seen that the drug becomes less effective and may also have side effects like dyskinesia, dementia and nausea. We choose these drugs because CCG4986, CCG-63802 and CCG-63808 are relatively selective for RGS4 over other R4 family members, including the closely related RGS8 and RGS16. In accordance with various other studies, it was observed that the binding of these small molecule RGS inhibitors to the RGS4 protein leads to conformational changes in the complex as seen through the changes in the Gq(alpha)–RGS–drug complexes' accessible surface area. To evaluate the change in binding energy because of the small molecule inhibitors on the RGS4–drug complex stability we carried out protein–ligand docking (Fig. 1(a), (b), (c)). The energy of the drug–protein complexes showed us that CCG63802 had the least energy, −6.00 followed by CCG63808 with −5.99 while the docking energy of the irreversible CCG4986 was computed as −5.10. This showed that these ligand molecules bind at the allosteric site of RGS4 to form the stable complex (Table 1). But according to the docking energy of these ligands with RGS4 we found that CCG63802 formed the most stable complex among the three drug molecules.

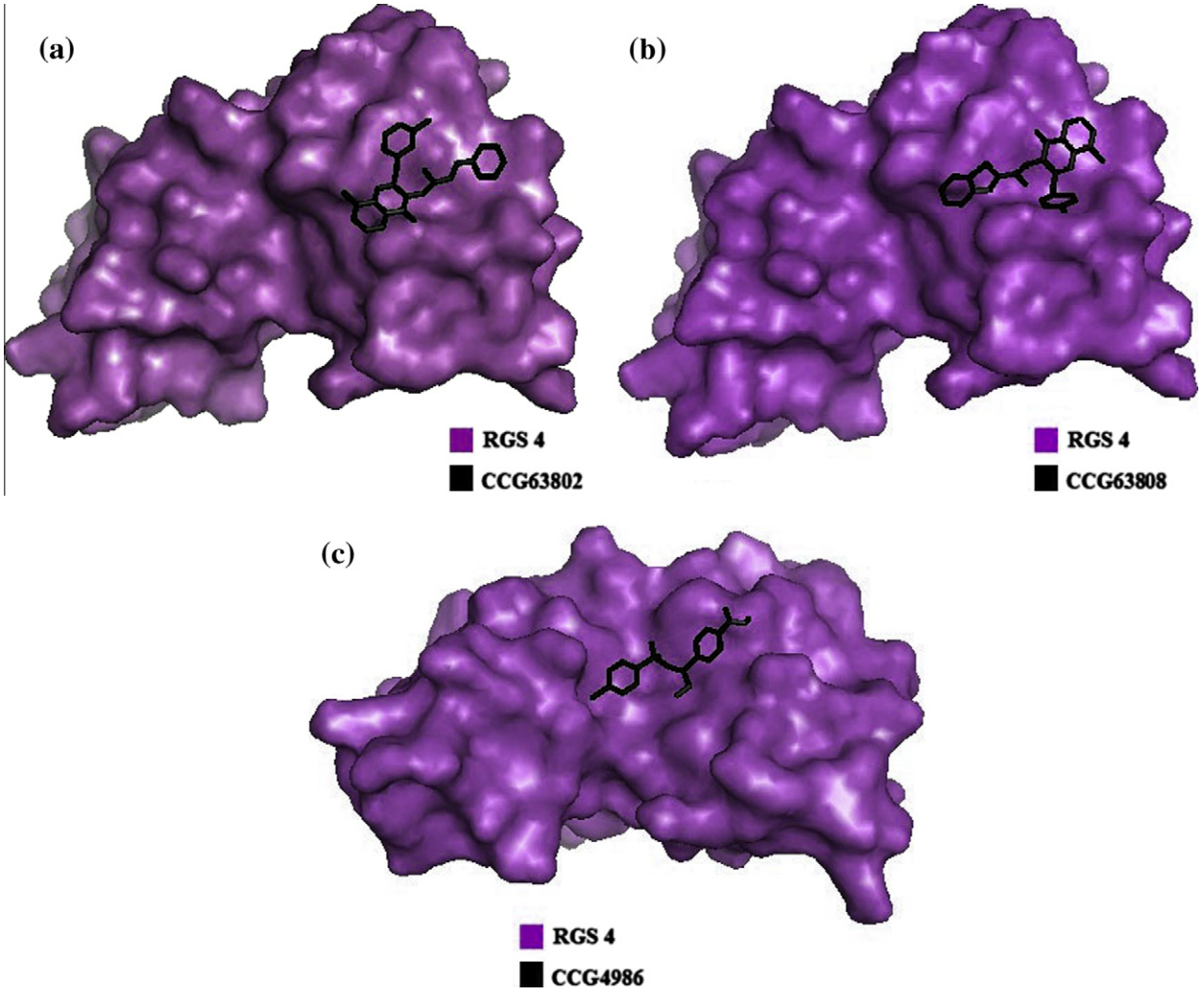

Figure 1 RGS4 complex with small RGS modulators, the binding sites for these drugs are present at the allosteric site; CYS 95, CYS132 and CYS 148. (a) The native RGS4 (light purple)- ccg638029(dark grey, stick model) (b) The native RGS4(light purple)- ccg63808(dark grey, stick model) (c) The native RGS4(light purple)- ccg4986 (dark grey, sick model).

Table 1 The docking energies of the Rgs and the drug(ccg63808,ccg63802, ccg4986) complexes.

Protein–drug	Docking energy
Rgs4–ccg63808	−5.99
Rgs4–ccg63802	−6.00
Rgs4–ccg4986	−5.10

Table 2 The total accessible surface area and docking energies of the Gq(alpha)–Rgs and the drug(ccg63808,ccg63802,ccg4986) bound complexes.

Complex	Docking energy	ASA(in A^2)
Gq(alpha)–Rgs4	−87.9 ± 4.7	21070.0
Gq(alpha)–Rgs4–ccg63808	−74.7 ± 4.1	20983.6
Gq(alpha)–Rgs4–ccg63802	−71.6 ± 1.4	20685.6
Gq(alpha)–Rgs4–ccg4986	−81.1 ± 4.1	20757.3

ASA = accessible surface area.
Rgs = Regulators of G Protein Signalling.

Then the protein–protein docking was carried out to find the energy required to bind the RGS4–drug complex to Gq(alpha) which led us to the stability characteristics of the complex (Table 2). The drugs which were studied in our paper inhibit the protein–protein interactions thus making the RGS4–Gq(alpha) complex less favourable. The protein–protein docking energy for Gq–RGS4 (alpha) was −87.9 ± 4.7 and the accessible surface energy was 21070.0. The docking energy for Gq(alpha)–RGS4–CCG63802 was −71.6 ± 1.4 and the accessible surface energy was 20685.6, this showed that there was a conformational change in the complex due to the binding of CCG63802 to RGS4. The surface energy changes to a more compact structure after docking with the RGS which is bound with the drug. For Gq(alpha)–RGS4–CCG63808 the docking energy was −74.7 ± 4.1 and the accessible surface energy was 20983.6, this showed that there was a conformational change in the complex when compared to the original structure. The surface energy changed to a more compact structure after docking with the RGS which is bound with the drug CCG63808 as seen in the previous drug. The RGS4–CCG4986 binds irreversibly to the Gq (alpha) protein with an energy of −81.1 ± 4.1 and the accessible surface energy was 20757.3, the conformational changes were in accordance to the previous drugs. The RGS4–drug complexes with the Gq(alpha) were less stable than the complex formed by the native RGS4,this was due to the conformational changes due to the drug binding at the allosteric site to RGS4. This showed us that the binding of the RGS4–drug complex to the Gq(alpha) protein was less favourable than the native RGS4. The change in conformation of the RGS4–drug complex is probably the result of the increase in flexibility, a possible reason for the decrease in the stability of the Gq(alpha)–RGS4–drug complex.

The matrix generated by elNémo displays the maximum distance fluctuations (Table 3) between all pairs of C alpha atoms and between the two extreme conformations that were computed for this mode.

The matrix for native RGS4 showed the largest decrease in fluctuations. The cumulative sum of largest increase was 17.32 and largest decrease was −17.46 where as the matrix for CCG-63802 bound RGS4 showed the largest increase in distance fluctuations. The cumulative sum of the largest increase was 20.17 and largest decrease was −12.95. Therefore this showed that CCG-63802 bound RGS4 is more flexible than the native form of RGS4. In the matrix for CCG-63808 bound RGS4 we could see that there is the largest decrease in distance fluctuations (Fig. 2(a), (b), (c) and (d)). The cumulative sum of largest increase was 12.82 and the largest decrease was −18.5.

Table 3 C-alpha fluctuation distance (in Å) for the interacting residues corresponding to the neighbouring residues in protein–protein complex.

Gq(alpha)–Rgs4 and Gq(alpha)–Rgs4–ccg63802 complex				
GLU 281	0.08	**GLU 83**	−0.08	ASN 336
LYS 125	0.14	**GLU 83**	−0.02	PHE 328
LYS 125	0.08	**TYR 84**	−0.10	ASN 336
LYS 125	0.18	**TYR 84**	−0.05	LYS 81
ASN 137	0.05	**ASN 128**	−0.06	ILE 62
GLU 280	0.13	**ASN 128**	−0.10	GLY 197
ASN 137	0.22	**ASP 163**	−0.06	PHE 339
ALA 342	0.06	**ASP 163**	−0.15	GLY 66
GLN 142	0.27	**ARG 167**	−0.07	PHE 339
THR 124	0.04	**ARG 167**	−0.06	THR 175
MET 284	0.26	**ARG 172**	−0.10	ALA 343
THR 124	0.07	**ARG 172**	−0.03	GLU 281
GLU 143	0.32	**LEU 175**	−0.12	ALA 343
ASP 69	0.05	**LEU 175**	−0.04	ARG 166
Gq(alpha)–Rgs4 and Gq(alpha)–Rgs4–ccg63808 complex				
GLU 281	0.08	**GLU 83**	−0.08	ASN 336
GLN 303	0.08	**GLU 83**	−0.10	ALA 343
LYS 125	0.08	**TYR 84**	−0.10	ASN 336
SER 68	0.06	**TYR 84**	−0.13	ALA 343
ASN 137	0.05	**ASN 128**	−0.06	ILE 62
SER 171	0.09	**ASN 128**	−0.15	GLN 303
ASN 137	0.22	**ASP 163**	−0.06	PHE 339
SER 171	0.12	**ASP 163**	−0.09	GLN 303
GLN 142	0.27	**ARG 167**	−0.07	PHE 339
ASP 69	0.12	**ARG 167**	−0.07	GLN 303
GLN 142	0.27	**ARG 172**	−0.10	ALA 343
GLY 66	0.11	**ARG 172**	−0.03	GLN 303
GLU 143	0.32	**LEU 175**	−0.12	ALA 343
GLU 281	0.09	**LEU 175**	−0.04	GLU 47
Gq(alpha)–Rgs4 and Gq(alpha)–Rgs4–ccg4986 complex				
GLU 281	0.08	**GLU 83**	−0.08	ASN 336
SER 85	0.11	**GLU 83**	−0.03	VAL 51
LYS 125	0.08	**TYR 84**	−0.10	ASN 336
LYS 125	0.18	**TYR 84**	−0.04	ALA 78
ASN 137	0.05	**ASN 128**	−0.06	ILE 62
GLU 280	0.12	**ASN 128**	−0.11	ALA 342
ASN 137	0.22	**ASP 163**	−0.06	PHE 339
ALA 342	0.04	**ASP 163**	−0.16	GLY 66
ASN 69	0.05	**ARG 167**	−0.08	GLY 66
GLN 197	0.04	**ARG 167**	−0.07	SER 118
GLN 142	0.27	**ARG 172**	−0.10	ALA 343
ALA 28	0.07	**ARG 172**	−0.03	GLU 281
GLU 143	0.32	**LEU 175**	−0.12	ALA 343
ASP 69	0.05	**LEU 175**	−0.03	TYR 124

ASP: aspartate; ALA: alanine; SER: serine; ILE: isoleucine; LEU: leucine; PHE: phenylalanine; ASN: asparagine; VAL: valine; ARG: arginine; GLU: glutamate; HIS: histidine; THR: tyrosine; TRP: tryptophan; CYS: cysteine; VAL: valine.

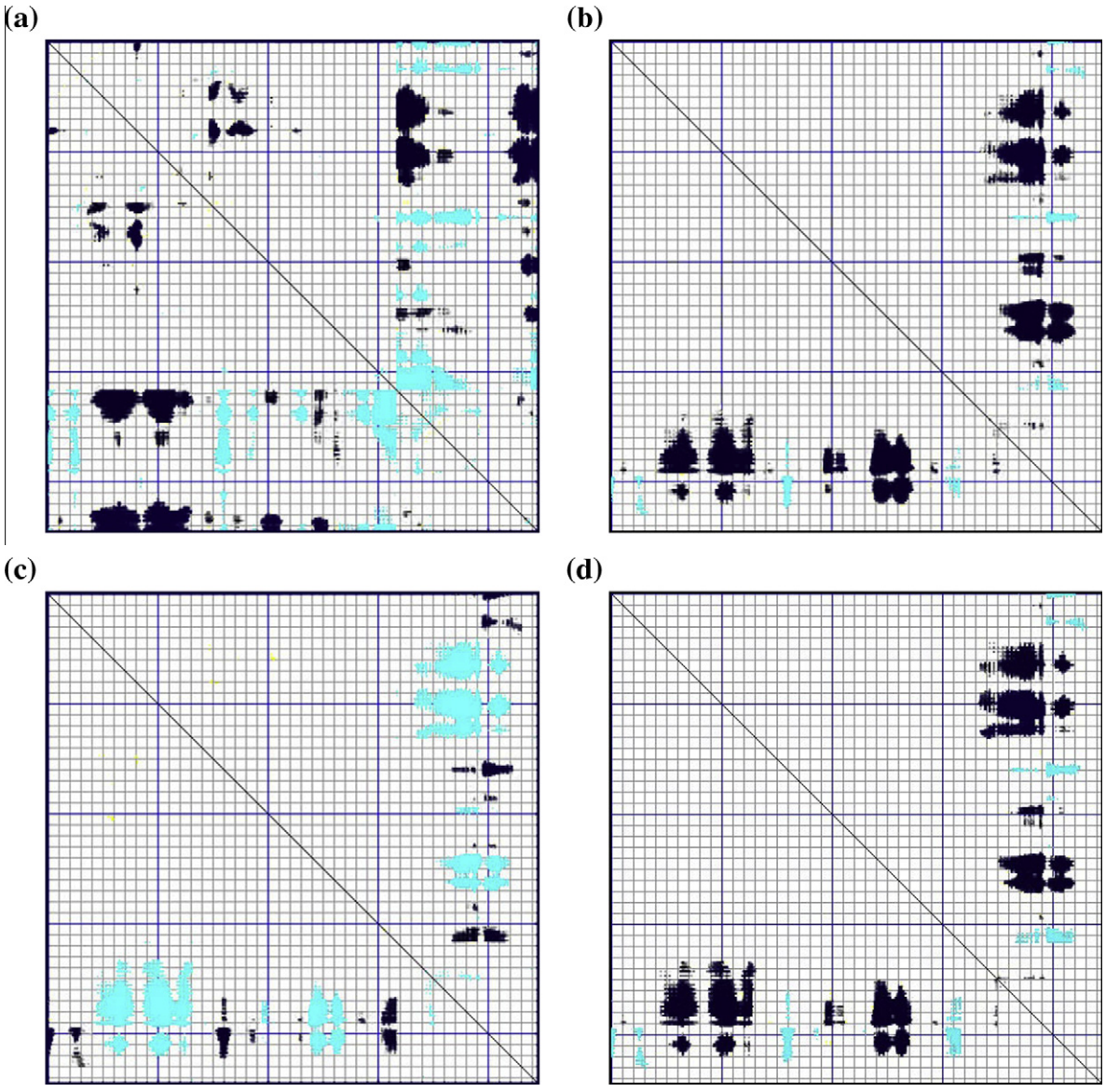

Figure 2 Distance fluctuation maps which highlight residue pairs i and j with the strongest variation in the distance between their C-alpha atoms in a given mode. Here the top distance fluctuations are coloured in dark grey and light grey. Distance fluctuations coloured in dark blue shows the increase in distance fluctuation whereas distance fluctuations coloured in light blue shows the decrease in distance fluctuation. Fig. 2(a), (b), (c) and (d) depict the distance fluctuation for Gq(alpha)–RGS4, Gq(alpha)–RGS4–ccg63802, Gq(alpha)–RGS4–ccg63808, and Gq(alpha)–RGS4–ccg4986 respectively.

Therefore this showed that like CCG-63802 bound RGS4 the CCG-63808 bound RGS4 is also more flexible than the native form of RGS4. Similarly in the matrix generated for CCG-4986 bound RGS4, it showed the largest increase in distance fluctuations. The cumulative sum of the largest increase was 20.27 and the largest decrease was −13.37. Therefore as seen in the results of the other two drugs the elNemo results for CCG-4986 bound RGS4 showed that there is more flexibility in CCG-4986 bound RGS4 than the native form of RGS4. This increase in flexibility in comparison to the native state could account for the change in the stability of RGS4 bound drug complex. But CCG4986 is irreversible and also is not functional in a reducing environment. CCG-63802 and CCG-63808, with their reversibility and activity in glutathione, a predominant intracellular reductant, represents a significant step forward in the development of RGS small molecule protein–protein interaction inhibitor (SMPPIIs) [34].

4. Conclusion

The results affirmed that CCG63802 is best suited over the other two drugs. It was seen that RGS4–CCG63802 complex was more stable than the other two complexes. The Gq(alpha)–RGS4–CCG63802 complex also showed that it was less favourable when compared with the other drugs. As it strongly binds to RGS4 and changes its conformation in such a way that it prevents the formation of the Gq(alpha)–RGS4–CCG63802 complex. This has been affirmed by our results and thus can be considered as a potential drug to treat Parkinson's by a non-dopamine treatment. CCG63802 binds to

RGS4 reversibly therefore it is preferred when compared with CCG4986 which binds irreversibly to RGS4. Further research on the effect of RGS4 on in cholinergic neurons and other parts of the striatal should provide us with the needed information. The actual physical interaction among the D2 and A2A receptors should also be studied to understand how these receptors control the striatal plasticity. The research in finding targeting novel steps in signal-transduction pathways has become an exciting field therefore the research on the RGS inhibitors and protein–protein interaction inhibitors in general provides exciting new opportunities. The knowledge about the interaction of the small molecule RGS inhibitors with its environment can provide valuable information about how the pathway changes its functions because of the inhibition of the RGS by these molecules. Further studies of the mechanism and structure–activity relationships for this compound class and translation to cellular and animal models of RGS function are currently being studied. This would provide a marked increase in the number of potential pharmacological targets based on the small molecule inhibition of the RGS thus controlling the signal-transduction in the cells.

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