



RESEARCH ARTICLE

BIO INFORMATICS

POSSIBLE BINDING MODES OF WITHAFERIN-A AND ITS ANALOGS IN THE ACTIVE SITE OF nNOS MOLECULAR DOCKING STUDIES**ZAVED AHMED KHAN*¹ AND ASIT RANJAN GHOSH²**

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ABSTRACT

Docking simulations were carried out with AutoDock 3.0, using the withaferin-A bound to nNOS (PDB ID: 3JWT) to define the binding pocket. The appropriate ligand targets were built in SYBYL 6.8 by modifying the molecular structure of withaferin-A which was extracted from the crystal structure. The withanolides 1–3 and 4–5 were formed using SYBYL program. Molecular docking study revealed that all the ligands are completely buried inside the aromatic gorge of nNOS, while compounds 1, 3, and 5 extend up to the catalytic triad. A comparison of the docking results showed that all ligands generally adopt the same binding mode and lie parallel to the surface of the gorge. The superposition of the docked structures demonstrated that the non-flexible skeleton of the ligands always penetrates the aromatic gorge through the six-membered ring A, allowing their simultaneous interaction with more than one subsite of the active center. The affinity of ligands with nNOS was found to be the cumulative effects of number of hydrophobic contacts and hydrogen bonding. The nNOS inhibitory potential could make compounds 1–5 possible drug candidates for further study to treat anxiety and associated problems.



KEYWORDS

Withanolides; *Withania somnifera*; nNOS; Molecular docking studies; Enzyme inhibition;

INTRODUCTION

The comprehensive study of the nNOS/inhibitor complexes by X-ray crystallography has indicated a nearly identical three-dimensional structure of the active site, located 20 Å from the protein surface at the bottom of a deep and narrow gorge¹. The different positions of the known inhibitors in the binding pocket suggest that more than one clearly defined binding site exists which are called esteratic and anionic subsites. Esteratic subsite contains catalytic triad (Ser200, His440, and Glu327)² and oxyanion hole forming residues (Gly118, Gly119, and Ala201)³. The quaternary ammonium-binding locus (Trp84, Phe330, and Glu199) is responsible for binding of the quaternary trimethyl ammonium tail group of nNOS by cation- π interaction⁴. The peripheral site, which is also called peripheral anionic site (PAS), includes Tyr70, Asp72, Tyr121, Tyr334, and Trp279 residues^{5,6}. Ligand occupation of the peripheral anionic site may allosterically change the conformation of the active center⁷. Aromatic residues lining the gorge and residues, located at the outer rim of the gorge have been postulated to be involved in the initial binding and guiding of the substrate towards the active site⁸.

The discovery of natural nNOS inhibitors has been a very challenging area of drug development due to the involvement of nNOS in anxiety and other related mood disorders. The steady-state inhibition kinetics, pharmacological profiles, SAR and 3D-QSAR, CoMFA and CoMSIA⁹ studies have been conducted on a plenty of compounds. Continuing our program to introduce new drug candidates of anxiety disorders, we identified analogs of withaferin-A 1–5, with efficacious nNOS inhibitory potential. The objectives of the current investigation were, first, to identify the nNOS inhibitors and then to explore the possible binding modes of these

compounds in the active site of nNOS by molecular docking studies.

MATERIALS AND METHODS

Molecular docking studies

The three-dimensional structures of ligands were constructed and optimized using the SYBYL program¹⁰. Energy minimization was performed using the tripos force field with a distance gradient algorithm with convergence criterion of 0.05 kcal/(mol Å) and maximum 1000 interactions, respectively. The FlexX¹¹ method was applied to dock ligands with most of the default parameters, in the aromatic gorge of nNOS complexed with L-NAME (PDB id; 3JWT). A radius of 6.5 Å was used to define the active-site interaction points. FlexX software is a fast and flexible algorithm for docking small ligands in binding sites of the enzymes, using an incremental construction algorithm that actually builds the ligands in the binding site¹². The software incorporates protein-ligand interactions, placement of the ligand core, and rebuilding the complete ligand. Docking results were analyzed by VMD¹³ and LIGPLOT¹⁴. All the computational studies were performed by using computer server on a dual processor 1.5 GHz Intel-based PC running the LINUX SUSE 8.2 (Kernel 2.4) operating system.

RESULTS

Autodock calculations

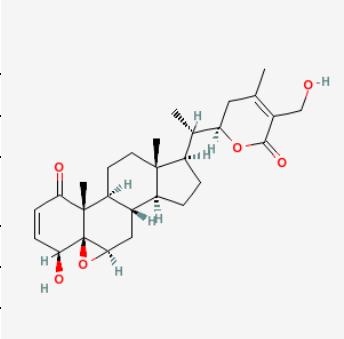
Docking simulations were carried out with AutoDock 3.0 using the withaferin-A¹⁵ bound to nNOS (PDB ID: 3JWT) to define the binding pocket. The appropriate ligand targets were built in SYBYL 6.8 by modifying the



molecular structure of withaferin-A which was extracted from the crystal structure. Energy minimizations were performed following both the addition of polar hydrogen atoms and partial atom charge calculations by the Gasteiger–Marsilli method¹⁶. Appropriate atom types were specified and labeled for physiological conditions, and ligand chirality was inspected. An atom fit between the ligand and the active site conformation of withaferin-A was performed, and

the center was defined. The grid box dimensions were set at 31 Å × 28 Å × 31 Å, and the spacing was set to 0.375 Å. For a preliminary identification of appropriate structures, a visual comparison was made of the superposition of the guanidino moiety of the calculated conformations, and that of withaferin-A. Conformations were then further evaluated based on their total docking energies.

Table 1
Inhibition constant of withaferin-A

Compounds	Ki Inhibition	Amino Acids
	Constant	
	23	Ile 444, Glu 199, Trp84, Ser200
		His44, Phe330, Phe331, Tyr121
		Trp279, Phe290

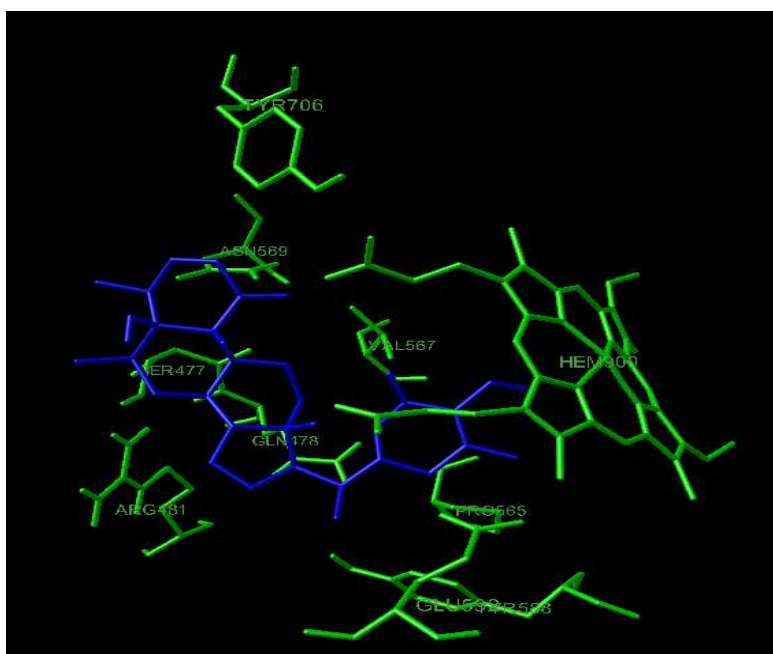


Fig .1
Withaferin-A and nNOS complex



Analogues of withaferin-a

In order to predict the interactions of compounds 1–5 in the aromatic gorge of nNOS, docking positions with the lowest energy were achieved, indicating that the phase space has been sufficiently sampled. Docking protocol for each ligand was repeated many times and best docking positions with their respective minimum energies were consistently reproduced. The size and shape of this series of ligands supported a gorge-spanning binding mode. Therefore, nNOS co-crystallized with L-NAME was taken as a model, for comparison in order to monitor the performance of our docking approach employed in this study.

The best ranking docking solutions showed that nNOS could accommodate compounds 1–5 ideally inside the aromatic gorge. Comparison of the docking results with aliphatic bis-quaternary inhibitors such as L-NAME and/or aromatic ring containing inhibitors such as BW284C51¹⁷ shows that compounds 1–5 could not penetrate deep into the aromatic gorge like these inhibitors, and rather remained closed to the anionic subsites. This might be due to the bulky skeleton of compounds 1–5 as compared to BW284C51 and L-NAME known inhibitors of nNOS. Compounds 1–5 orient themselves along the active-site gorge in such a way that their activity can be attributed only to different substituents at these ligands. Like DME999¹⁸ compounds 1–5 span the entire nNOS surface with possibility of multiple-binding sites. The superimposition of the L-NAME ligand in the crystal structure of the complex (PDB entry 3JWT) with our results shows that L-NAME enters relatively deeper into the aromatic gorge than our new inhibitors. A comparison of the docking results of all five ligands showed that all compounds generally adopt the same binding mode. This similar binding mode is not surprising, as all the ligands have almost same structure with minor difference of functional groups. The superposition of the docked structures clearly demonstrates that the non-flexible skeleton of the ligands always penetrates the aromatic gorge through the six-membered ring A. Thus, ring A is placed at the bottom of the

gorge that might be due to the apparently greater hydrophobicity of ring A as compared to the five-membered ring D. The compounds 1–5 under study were found to be completely buried inside the aromatic gorge of the nNOS. This position contributes to the stabilization of the nNOS-ligand complexes, as the backbones of the ligands are highly hydrophobic due to their carbocyclic character. The principal interactions holding the ligands 1–5 in the active site of nNOS are summarized as follows.

COMPOUND 1–nNOS COMPLEX

Ligand 1 has interaction with all the four subsites of the active center of nNOS. Ser200 forms hydrogen bond (3.0 Å) with the carbonyl oxygen of ring A. Similarly, side chain carbon has hydrophobic contact with ring A. In addition to hydrogen bonding, compound 1–nNOS complex is also stabilized by the hydrophobic contacts with PAS (Tyr121, Trp279) residues of nNOS. Trp279, located at the top of the gorge, is thought to have the ability to regulate entry of ligand 1 into the gorge. Furthermore, compound 1 also holds hydrophobic interactions with acyl-binding locus (Phe290, Phe331) and choline-binding site (Trp84, Glu199, and Phe330). In case of L-NAME only amino acid residues of Phe331, His440, Gly441, Trp84, Trp279, and Tyr70 of nNOS have hydrophobic contacts with the ligand. BW284C51 and E2020 bind through their two-phenyl and quaternary amino end-groups complexed to Trp84 and Phe330 (choline-binding). The affinity ($K_i = 23.0 \pm 0.1 \mu\text{M}$) of compound 1 with nNOS may be the cumulative effect of hydrogen bonding along with hydrophobic contacts, with all the subsites of the active center due to the interactions with all the four subsites of the active-site mixed-type of inhibition exhibited by ligand 1 is not unexpected.

COMPOUND 2–nNOS COMPLEX

Ligand 2 binds at anionic subsites and thus hinders access to esteratic site. Amino acid residues of PAS and choline-binding pocket of nNOS play a key role in the



stabilization of compound 2–nNOS complex. Tyr70 forms hydrogen bond (3.2 Å) with carboxyl group oxygen of ring B, while residues of Tyr121, Tyr334, and Tyr279 interacted with the ligand through hydrophobic interactions. Similarly, Tyr130 in choline-binding region forms hydrogen bond (2.8 Å) with methoxy oxygen of ring E, while Trp84 has π – π stacking that is energetically favorable to stabilize compound 2–nNOS complex. Similarly, Glu199 is also strongly involved in hydrophobic contacts. This residue is assumed to make concave type of binding site that recognizes the quaternary ammonium function. Gly117 and Gly118 can form peptide bonds with both compound 2–nNOS and free nNOS. The oxyanion hole formed by the backbone NH groups of Gly118, Gly119, and Ala201 plays a critical role in stabilizing the structures of the transition states and lowers the activation barriers of nNOS catalyzed ATCh hydrolysis. An interesting observation seen in compound 2–nNOS complex is the flip of the peptide bond between Gly117 and Gly118. As a consequence, the position of the main chain nitrogen of Gly118 in the oxyanion hole is occupied by the carbonyl group of Gly117. This renders the oxyanion hole less accessible to substrate and less capable of stabilizing its tetrahedral intermediate for nucleophilic attack by Ser200. Furthermore, the flipped conformation can be stabilized by Gly117 that forms hydrogen bonds with Gly119 and Ala201, suggesting that the flip of the Gly117–Gly118 peptide bond should be an intrinsic property of nNOS. Compound 2 ($K_i = 20.8 \pm 0.2 \mu\text{M}$) has a strong binding with the nNOS as compared to compound 1 ($K_i = 23 \pm 0.1 \mu\text{M}$) that may be due to the strong hydrogen bonding and hydrophobic contacts in compound 2–nNOS complex.

COMPOUND 3–nNOS COMPLEX

Compound 3–nNOS complex is stabilized through the interactions involving all the four subsites of the active center. Ser200 (catalytic residue) forms hydrogen bond (2.8 Å) with carbonyl oxygen of ring A. PAS (Tyr121, Trp279) has hydrophobic contacts with rings D and E near the entrance of the active site. Choline-

binding pocket amino acid residues (Trp84, Glu199, and Phe330) interact near the bottom of the gorge while acyl-binding locus (Phe331) interacts with C and side chain methyl group near the center of the gorge, respectively. As previous study has revealed that proper positioning of the Glu199 carboxylate relative to the catalytic triad can play a key role in defining its functional role in the interaction of nNOS with substrates and inhibitors. In complexes involving compounds 1 and 3, Glu199 has hydrophobic interaction in the vicinity of catalytic residues near the bottom of the gorge. While in the compound 2–nNOS complex, it has interaction with ring E near the entrance of the gorge of nNOS. That may be favorable for the higher activity of the compound 2–nNOS complex. The low inhibitory potential of compound 3 ($K_i = 45.0 \pm 0.01 \mu\text{M}$) than 2 ($K_i = 20.8 \pm 0.2 \mu\text{M}$) may be attributed due to less hydrophobic contacts and hydrogen bonding in compound 3–nNOS complex than in compound 2–nNOS complex.

COMPOUND 4–nNOS COMPLEX

Tyr121 is forming hydrogen bonds with epoxide oxygen (3.1 Å) at ring B and hydroxyl group (2.6 Å) present at the junction of rings A and B. PAS residues of nNOS (Asp72, Tyr334, and Trp279) are involved in hydrophobic contacts. Similarly, compound 4 has hydrophobic interactions with residues of the acyl-binding locus (Phe290 and Phe331) and choline-binding pocket (Phe330) near the bottom and middle of the gorge, respectively. Unique feature of compound 4–nNOS complex is that epoxide oxygen forms hydrogen bond with Tyr121, while in other nNOS–ligand complexes, reported in this article, no such type of bond formation with epoxide has occurred. This may be due to the fact that in compound 4–nNOS complex epoxide is present at position C-6/C-7 of ring B, having α -orientation and due to less operational steric hindrance it can form hydrogen bond easily, while in other nNOS–inhibitor complexes it is β -oriented and cannot easily form hydrogen



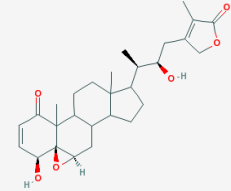
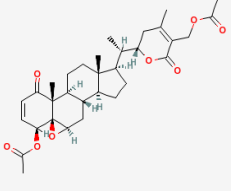
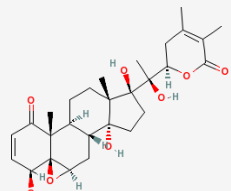
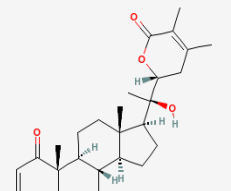
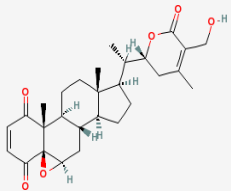
bonds with the amino acid residues present in the vicinity. Same is true with the hydroxyl group present at the junction of the rings A and B in ligand 4. Similarly, interactions with the Asp72 were observed only in compound 4–NNOS complex. Asp72 of nNOS located at the boundary between the PAS and the acylation site (active site) is a key residue with which the ligands can interact. Previous study revealed that Asp72 contributes very little to electrostatic effect to the cationic ligands²⁰. However, experiments of kinetics did not provide the atomic details for the role Asp72 plays when compound 4 enters or leaves the active-site gorge. As a negatively charged residue at the entrance of the deep gorge, Asp72 may generate electrostatic field affecting the cationic substrates or inhibitors. Asp72 does not often directly contact with these ligands, but can form water bridges with ligands leaving and entering the nNOS-binding gorge acting as clamp to compound 4 and place into the active site²¹. The lower inhibitory potential ($K_i = 40.5 \pm 0.05 \mu\text{M}$) of compound 4 may be due to the less hydrophobic contacts although Tyr121 makes two hydrogen bonds which may be due to unfavorable conformational changes of the ligand with the receptor.

COMPOUND 5–nNOS COMPLEX

Compound 5–nNOS complex is stabilized through the interactions with all the four subsites of the active center resulting in the mixed-type of inhibition, which is in agreement with steady-state inhibition data. In compound 5–nNOS complex, carbonyl oxygen of the ligand at ring A forms strong hydrogen bonds with catalytic triad residues Ser200 (2.0 Å) and His440 (3.0 Å). Compound 5 also experiences hydrophobic interactions near the entrance of the cavity with PAS (Trp279, Tyr334) residues of nNOS. Choline-binding pocket (Phe330, Trp84) and acyl-binding locus (Phe331) residues also have hydrophobic contacts with ring C and chain methyl group side chain of ligand 5. The higher inhibitory potential ($K_i = 20.0 \pm 0.1 \mu\text{M}$) of compound 5 may be due to strong hydrogen bonding and hydrophobic interactions. Due to the interactions with all the four subsites of the active center of nNOS, mixed-type of inhibition is predicted.

Therefore, compounds 1–5, PAS binding and with catalytic triad-binding nNOS inhibitors, might represent a new therapeutic option, as these compounds should be able to overcome the anxiety disorders.

Table - 2
Structures and results of docking studies

Compounds	Ki Inhibition Constant	Amino Acids
	23	Ile 444, Glu 199, Trp84, Ser200 His44, Phe330, Phe331, Tyr121 Trp279, Phe290
	20.08	Trp279, Tyr70, Tyr334, Tyr21 Phe331, Trp84, Gly117, Gly118 Ile444, Glu199, Tyr130
	45	Gly117, Ser200, Ile444, Glu199 Trp84, Phe330, Phe331, Tyr121 Trp279
	40.5	Asp72, Phe330, Phe331, Phe290 Tyr334, Trp279
	20.5	Gly117, His440, Gly118, Trp84 Ser200, Phe330, Phe331, Tyr334 Trp279

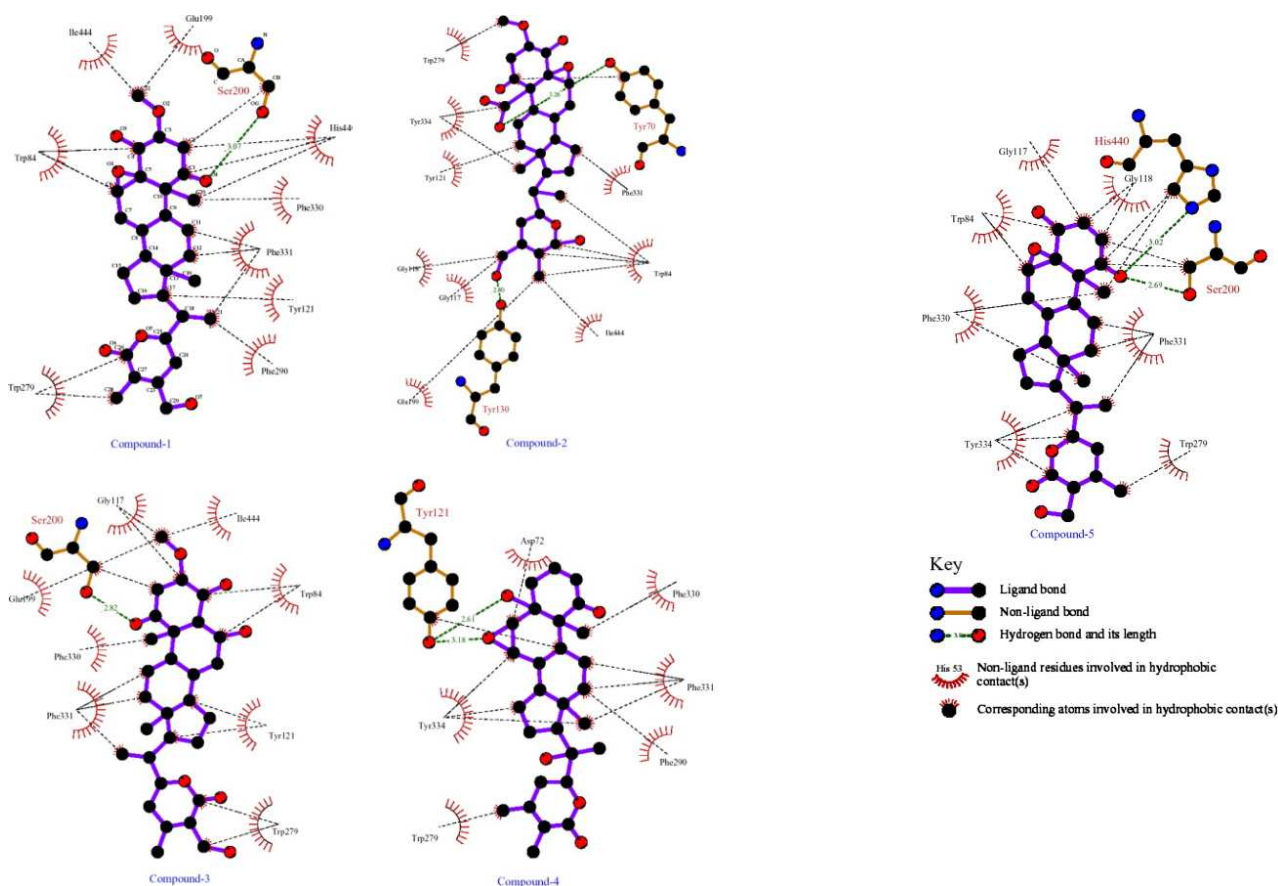


Fig. 9.2

2D-Schematic representation of compounds 1–5 by LIGPLOTS, showing that hydrophobic contacts and hydrogen bonding are the principal interactions, holding the ligand–receptor complexes in stable form.

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