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Research Article

Identification of Withanolide-M and Stigmasterol as Potent neuroprotectant and Dual inhibitor of Inducible/Neuronal Nitric Oxide Synthase by Structure-Based Virtual Screening

Gaurav Kumar*, Sumedha Mukherjee, Ranjana Patnaik

School of Biomedical Engineering, Indian Institute of Technology (Banaras Hindu University), Varanasi-221005, UP, India

*gaurav.rs.bme14@iitbhu.ac.in

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Abstract: Nitric oxide (NO) is an important intracellular signaling molecule, generated by catalysis from nitric oxide synthases (NOSs). In the face of NO playing beneficial roles, its overproduction by the neuronal nitric oxide synthase (nNOS) or inducible nitric oxide synthase (iNOS) is detrimental in neurological disorders, whereas that derived from the endothelial nitric oxide synthase (eNOS) is beneficial. Therefore, dual inhibition of iNOS and nNOS without inhibiting eNOS is a promising neuroprotective approach for combating stroke. *Withania somnifera* (WS) has been used for centuries as a nerve tonic and Nootropic agents in Ayurveda. Present structure-based molecular docking study was performed to identify novel, potent and dual iNOS/nNOS inhibitor by screening the *Withania somnifera* constituents. A ligand database containing 36 phytochemicals present in *W. somnifera* along with nNOS and iNOS selective inhibitor was molecularly docked onto catalytic heme domain of three NOS isoforms. This approach identified two phytosteroids withanolide-M and stigmasterol that have higher selectivity, bind with the lower binding energy and established a number of H-bonds or hydrophobic contacts with the catalytic oxygenase domain of iNOS and nNOS than their selective inhibitors (AT2 and S19). Their suitability for Lipinski's Rule of Five, the ability to cross the Blood-Brain Barrier (BBB), high human intestinal absorption score make them a potent possible neurotherapeutic agent to combat neurological disorders mediated by nNOS and iNOS activation.

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INTRODUCTION

Nitric oxide (NO) is an important intracellular signaling molecule, generated by catalysis from nitric oxide synthases (NOSs). In Mammals, three isoforms of nitric oxide synthase (NOS) have been identified: neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2) and endothelial NOS (eNOS or NOS3). These NOS enzymes are homodimeric proteins consisting an N-terminal catalytic oxygenase domain and a C-terminal reductase domain that are linked through a calmodulin-binding motif. N-terminal oxygenase domain contains binding sites for heme, a cofactor site for tetrahydrobiopterin (H4B), and arginine (substrate), whereas the C-terminal reductase domain provides binding motifs for FAD, NADPH, and FMN. The two-step oxidation of L-arginine carried out by three isoforms produce NO and L-citrulline (fig. 1). NO produced by eNOS and nNOS play a vital role in the relaxation of smooth muscles, and neurotransmission respectively, and iNOS is expressed in response to cytokines or pathogens to kill bacteria, viruses, and tumor cells [1].

In the face of NO playing beneficial roles, its overproduction by the neuronal or inducible isoform of nitric oxide synthase (nNOS, iNOS) is detrimental in several neurological disorders [2-4], whereas that derived from the endothelial isoform is beneficial [5]. Therefore, dual inhibition of iNOS and nNOS without inhibiting eNOS is a promising neuroprotective approach for combating neurological disorders. Studies suggested that nNOS and iNOS inhibitors significantly reduced infarct volume in both permanent and transient models [6]. Moreover, the catalytic site structure of nitric oxide synthases has the high degree of similarity that poses an obstacle to the isoform selectively. However, X-ray diffraction and NMR structures of NOSs with various ligands provided insights into the catalytic mechanism and mode of binding [7]. These findings provide sufficient information regarding the interaction between the active site residues and ligands hence can be utilized to design potent and selective drug-like NOS inhibitors. *Withania somnifera* (WS) or Ashwagandha, popularly known as Indian Ginseng, belongs to the family Solanaceae. It has been used for centuries as a nerve tonic and nootropic agents in Ayurveda [8]. Previously many studies

have reported that plant extract of WS confers neuroprotection by inhibiting both AChE and nNOS [9] and attenuate lipid peroxidation in the striatum and cortex of the brain [10]. Therefore, present structure-based molecular docking study was designed to identify novel, potent and dual iNOS/nNOS inhibitor by screening the *W. somnifera* constituents.

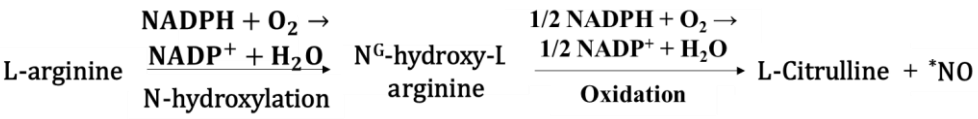

Fig. 1 Scheme of the two-step oxygenation of arginine catalyzed by NOSes.

METHODS

Structure of nitric oxide synthase isoforms

The three-dimensional crystal structure of three isoforms of human NOS: iNOS (PDB id: 4CX7), nNOS (PDB id: 4D1N) and eNOS (PDB id: 4D1O) were downloaded from RCSB-Protein Data Bank (PDB). These structures of heme domain were determined by X-ray diffraction method and deposited to the PDB by Li *et al.* [11, 12]. Table 1 presents the additional information about three isoforms used for the present study.

Selection and retrieval of ligands structure

A ligand database containing 36 phytochemicals present in *W. somnifera* used for this study were previously reported by our lab [13-15]. Their drug-likeness/ Lipinski's Rule of Five (LRo5), the prediction for Blood Brain Barrier (BBB) penetration ability, human intestinal absorption (HIA) score, and mutagenicity was previously reported [8, 13-15]. The 3D structure of these compounds was downloaded in .sdf format from NCBI-PubChem database. The structure of nNOS selective inhibitor- (S)-3-[(2-amino-4-methylquinolin-7-yl)methoxy]-5-(2-(methylamino)propyl)benzonitrile (S19) and iNOS selective inhibitor- Ethyl 4-[(4-Methylpyridin-2-yl)Amino]Piperidine- 1-Carboxylate (AT2) were downloaded from RCSB-Protein Data Bank PDB ID: 5UO7 [16] and PDB ID: 3E7G [1] respectively.

Docking preparation for ligands and three isoforms of NOS

All the phytochemicals and nNOS/iNOS selective inhibitors were converted from .sdf to .pdb format and then energy minimized by applying mmff94 force field and conjugate gradients optimization algorithm for 200 steps using PyRx - Python prescription 0.8 [17]. UCSF-Chimera [18] performed the docking preparation for the enzymes. We kept only chain B of the three isoforms for molecular docking study, and other chains were removed from their crystal structures. The chain B further refined by removing all heteroatoms and ligands (except H4B and heme). Further, the addition of hydrogens and deletion of solvent were performed. Atoms in standard residues are assigned charges and atom types taken from Amber force field (AMBER ff14SB), and nonstandard residues are directly assigned their net charges with semi-empirical (AM1) with bond charge correction (BCC) (AM1-BCC) and are computed using ANTECHAMBER [19]. Successively, minimization of energy was performed for three isoforms by steepest descent method with 100 steps and conjugate gradient method with ten steps (step size 0.02 A°).

Molecular Docking Simulation procedure

The dock ready 3D atomic coordinates for the catalytic heme domain (Chain B) of three isoforms were loaded on Auto Dock Tools 1.5.6 (ADT) [20]. The grid parameter file (.gpf) and docking parameter file (.dpf) were generated for each ligand. A grid box with the number of points 100, 80 and 110 in X, Y and Z dimensions, 0.375Å spacing, centered on macromolecule for X, Y and Z coordinates and having enough space for the ligands movement was generated. Rigid docking was performed with 30 independent runs (ga_run) by using the Lamarckian genetic algorithm search parameters. The grid parameter file (.gpf) and docking parameter file (.dpf) were converted into the grid log file (.glg) and docking log file (.dlg) by using Cygwin DLL version 2.8.1. Further docking data is analyzed with Auto Dock Tools, and LigPlot+ (v.1.4.5) [21] is used for the visualization of hydrogen bonds and hydrophobic interaction established by ligand with heme domain residues.

Table 1. PDB 3D crystal structures of catalytic heme domain used in present study

PDB ID	NOS isoform	Expression system	Resolution	Chains	Amino acids
4CX7	iNOS	*E. coli*	3.16 Å	A, B, C, D	431 residues (74-504)
4D1N	nNOS	*E. coli*	2.03 Å	A, B, C, D	420 residues (302-721)
4D1O	eNOS	*E. coli*	1.82 Å	A, B	440 residues (41-480)

RESULTS AND DISCUSSION

Inhibition potential analysis of ligands

To predict the binding conformation of nNOS selective inhibitor (S19), iNOS selective inhibitor (AT2), and 36 WS phytochemicals docking was performed into the catalytic heme domain of each NOS isoform. Out of 36 WS phytochemicals, only seven phytochemicals namely Anaferine, Anahygrine, Cuscohygrine, Stigmasterol, Withanolide A, Withanolide M and Withanone have shown the lowest binding energy for nNOS and iNOS as compare to the eNOS oxygenase domain. Table 2 summarizes the results of molecular docking studies comprising lowest binding energy, inhibition constant and selectivity over eNOS. Among seven

phytochemicals Anaferine, Anahygrine, Stigmasterol, Withanolide A, Withanolide M and Withanone have the higher selectivity for nNOS and only two compounds Stigmasterol, and Withanolide M have the higher selectivity for iNOS as compare to S19 and AT2 inhibitors respectively. Moreover, Stigmasterol and Withanolide M have the higher e/i and e/n selectivity than iNOS and nNOS selective inhibitors.

Table 2: Molecular docking scores and selectivity of screened ligands

Ligands	eNOS		iNOS		nNOS		Selectivity over eNOS	
	LBE	Ki (nM)	LBE	Ki (nM)	LBE	Ki (nM)	e/i	e/n
nNOS inhibitor								
S19	-9.19	182.34	-10.25	30.89	-9.70	78.16	5.9	**2.33**
iNOS inhibitor								
AT2	-7.90	1630	-8.70	417.09	-8.09	1170	**3.91**	1.39
***W.somnifera* Phytochemicals**								
Anaferine	-10.33	26.83	-10.51	19.85	-11.09	7.48	1.35	**3.59**
Anahygrine	-9.04	237.96	-9.38	132.74	-9.61	90.90	1.79	**2.62**
Cuscohygrine	-8.42	675.87	-8.56	535.71	-8.73	395.97	1.26	1.71
Stigmasterol	-9.50	109.56	-11.43	4.18	-10.00	46.57	**26.21**	**2.35**
Withanolide A	-10.10	39.40	-10.47	21.00	-11.59	3.17	1.88	**12.43**
Withanolide M	-10.51	19.64	-11.43	4.18	-11.08	7.51	**4.7**	**2.62**
Withanone	-10.15	36.54	-10.87	10.79	-11.19	6.33	3.39	**5.77**

LBE- Lowest Binding Energy in kcal/mol; Ki- Estimated Inhibition Constant in nM; Ki calculated from binding energy (kcal/mol) by Autodock 4; e/i and e/n are the selectivity ratios of Ki (eNOS) to Ki (iNOS/nNOS); S19: 3-[(2-amino-4-methylquinolin-7-yl)methoxy]- 5-[(2S)-2-(methylamino)propyl]benzonitrile; AT-2: Ethyl 4-[(4-methylpyridin-2-yl)amino]piperidine- 1-carboxylate.

Binding interaction analysis

Stigmasterol and Withanolide-M are further analyzed for their binding interaction with catalytic heme domain residues and compared with selective inhibitors of nNOS/iNOS (S19/AT2). Figure 2 shows the binding interactions of lowest binding energy conformation of S19, AT2, Stigmasterol, and Withanolide-M with the catalytic oxygenase domain of nNOS and iNOS. Lowest binding energy conformation of withanolide-M, stigmasterol, AT-2 and S19 with nNOS and iNOS catalytic domain were selected for interpretation of molecular docking results and visualized using LigPlot+ v.1.4.5.

The nNOS selective inhibitor (S19) formed two H-bonds with O1A and O2A atoms of Hem750 and one H-bond with N-atom of Gln483 as well as nine Hydrophobic contacts with Pro570, Tyr593, Glu597, Arg601, H4b760, Trp683, Arg608, Asp602 and Val572 residues of nNOS heme domain. The iNOS selective inhibitor (AT2) established two H-bonds with Tyr373 and Ala351 residues whereas it formed ten hydrophobic contacts with Glu377, Hem550, Trp372, Gly371, Asn370, Phe369, Val352, Pro350, Tyr347 and Gln263 residues of iNOS catalytic heme domain. Withanolide-M a phytosteroid formed three H-bonds with Hem750, Tyr711, and Arg486 residues and has ten hydrophobic contacts with nNOS catalytic heme domain residues. Withanolide-M and S19 have some similar interactions with Glu597, Gln483, Tyr593, Asp602, Trp683, and Val572 residues. Whereas, it formed two H-bonds with Tyr373 and three H-bonds with Asp382, Arg388, and Arg266 residues and established ten hydrophobic contacts with iNOS catalytic heme domain residues. When compared with iNOS selective inhibitor AT2 it has Tyr347, Hem550, Glu377, Phe369, Pro350, Val352, Ala351, Gln263 residues in similar interactions. Stigmasterol formed only one H-bond with Asp605 residue and established fifteen hydrophobic contacts with nNOS heme domain. It has interacted with some residues (Arg608, Asp602, Arg601, Tyr593, Glu597, Pro570, Hem750, Val572, Gln483 and H4b760) that are also present in the hydrogen bonding and hydrophobic interactions of the S19-nNOS complex. In the catalytic pocket of iNOS, stigmasterol interacted by forming two H-bonds with Asp385 and Arg381and thirteen hydrophobic contacts. The iNOS catalytic pocket residues Glu377, Hem550, Glu263, Gly371, Asn370, Ala351, Val352, Phe369, Pro350, and Tyr373 have equally interacted with stigmasterol and AT2 inhibitor.

Molecular docking study revealed that withanolide-M and stigmasterol have bound to the catalytic heme domain of iNOS and nNOS with the lower binding energy (the higher affinity) and the higher selectivity than their inhibitors. Both phytochemicals have shown similar binding conformations as demonstrated by AT2 and S19 (fig. 2) and established a number of hydrogen bonds and hydrophobic contacts with the catalytic site of nNOS and iNOS. Moreover, they bound with the lower binding energy and possesses the higher selectivity for the catalytic oxygenase domain of iNOS and nNOS compare to eNOS make them a dual inhibitor of iNOS and nNOS. Therefore, on the basis of lower binding energy, lower Ki value, establish a number of hydrogen bonds or hydrophobic contacts with catalytic pocket, comply with Lipinski's rule of five [13], able to cross the blood-brain barrier (BBB) [14] and high human intestinal absorption score [13, 14] we can say that withanolide-M and stigmasterol can be developed as a potent possible neurotherapeutic agent to combat neurological disorders mediated by nNOS and iNOS activation.

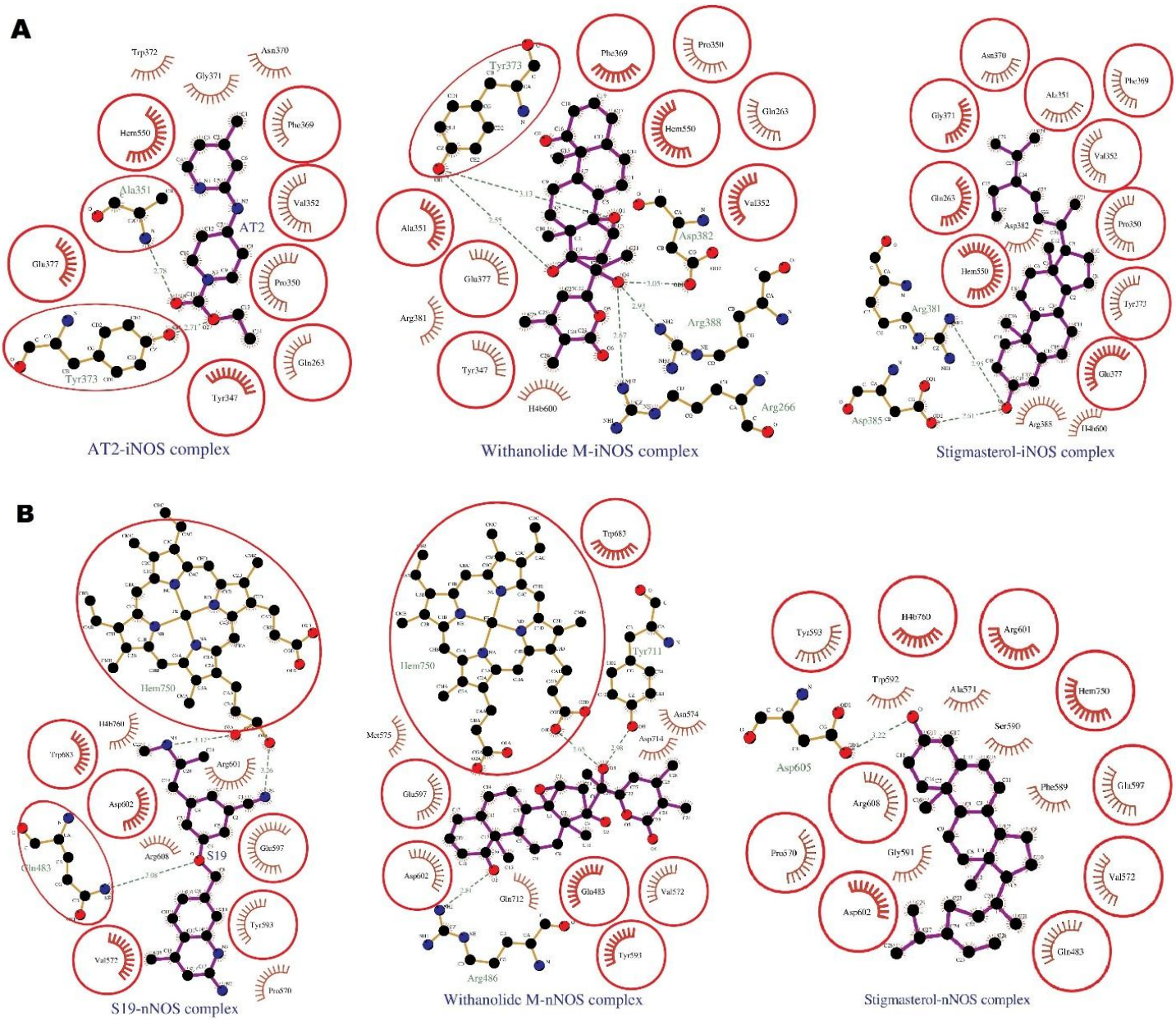

Fig. 2 Hydrogen bonding and hydrophobic interactions (visualization using LigPlot+ v.1.4.5) (A) the interactions of catalytic domain residues of iNOS with AT2, withanolide-M, and stigmasterol and (B) nNOS catalytic domain residues interaction with AT2, withanolide-M, and stigmasterol. (Ligand and residue bonds are blue and orange, respectively. H-bonds are shown with green dashed lines, and corresponding interatomic distances are indicated. The catalytic heme domain residues involved in the formation of hydrophobic contacts with the ligands are symbolized as labeled arcs with radial spokes that pointing to the ligand atoms with which they interact. Residues involved in similar H-bonds and hydrophobic interactions are shown in red circles.)

CONCLUSION

At the end of the screening, we predicted that among 36 phytochemicals, seven compounds were appearing as dual selective inhibitors for nNOS and iNOS over eNOS. Withanolide-M and Stigmasterol have shown the higher selectivity for iNOS and nNOS than their selective inhibitors (AT2 and S19). Both phytochemicals have shown similar binding conformations as demonstrated by AT2 and S19 and established a number of hydrogen bonds and hydrophobic contacts with the catalytic site of nNOS and iNOS. Therefore, on the basis of lower binding energy, lower Ki value, higher H-bonds formation or hydrophobic interaction with heme domain residues, suitability for Lipinski's Rule of Five and BBB penetration ability, we can hypothesize that Withanolide-M and Stigmasterol can inhibit nNOS and iNOS similarly as S19 and AT2 inhibitors. Both compounds can be developed as a neurotherapeutic drug with least risk of side effects to combat neurological disorders mediated by nNOS and iNOS activation. Further, to validate this hypothesis, their BBB penetration ability and dual selectivity for nNOS and iNOS over eNOS will be performed either in vivo or in vitro condition.

COMPETING INTERESTS: The authors have declared that no competing interests exist.

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About Author

Mr. Gaurav Kumar is doing his Ph.D. studies in the Biomedical Engineering at the Indian Institute of Technology (Banaras Hindu University), Varanasi. He obtained his Bachelors and Master Degrees in Biotechnology from Shobhit University, Meerut. Subsequently He joined CSIO-CSIR as Research Trainee. Hitherto he served as Assistant Professor, Shobhit University, Gangoh, Saharanpur; Shobhit University, Meerut and Lecturer at Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut. He has published several research papers in SCI-indexed Journals and Conferences of repute. His research interests including Stroke and neuroprotection, Drug designing and Neurophysiology.