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De Novo Design of Potent and Selective Neuronal Nitric Oxide Synthase (nNOS) Inhibitors by a Fragment-Based Approach

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Abstract: Nitric oxide, a gaseous free radical molecule (NO) behaves, as a secondary messenger in various tissues. It is responsible for different physiological functions and pathological symptoms. Mammals contain three different nitric oxide synthase (NOS) isoforms: neuronal NOS (nNOS: in the brain, in peripheral nervous system and muscle tissues), inducible NOS (iNOS: in macrophage cells), endothelial NOS (eNOS: in endothelial cells). Under certain pathological conditions and/or after certain ages excessive NO produced in brain causes tissue damage and oxidative stress. It also reacts with other free radicals to create specific molecular modifications. The excessive production of NO, especially by nNOS (in brain) is implicated in various disease states such as neurodegeneration, stroke, migraine and Parkinson's, Alzheimer's, and Huntington's diseases. The active sites of three NOS isoforms show great similarity; therefore, designing of selective nNOS inhibitors is not an easy task. The computational results carried out with all of the docking tools clearly demonstrate that the selected scaffold is a potential candidate for further modifications and optimization for designing selective and potent nNOS inhibitors. Subtle differences in the conformations of amino acid sequences (e.g. ASP597 in nNOS) of the three isoforms in the active site region were the determining factors for the selectivity and the potency of the compounds. In this study several hundred compounds were screened *in silico* using the ZINCv12 lead library for prioritization of lead candidates. *De novo* design method was used rationally for the modifications of selected scaffold within a target-binding site in order to enhance its binding affinity and selectivity to nNOS enzyme. The potency and the selectivity of nNOS isoform were achieved by introducing 1-methyl amino group at the forth position of the imidazole moiety of the best inhibitor. The positively charged 1-methyl amino group makes three hydrogen bonds with the two-propionate groups of the heme cofactor, which does not occur in eNOS and iNOS. Removing of 1-methyl amino group from scaffold totally abolished both potency and selectivity for nNOS. Newly designed inhibitor **7** shows nNOS inhibition 23 and 17 fold better than both eNOS and iNOS, respectively.

Keywords: nitric oxide synthase; in silico design; selective nNOS inhibitors; de novo design

1. Introduction

Nitric oxide a gaseous free radical molecule (NO) behaves as a secondary messenger in various tissues and is responsible for different physiological functions and pathological symptoms [1] Nitric Oxide syntheses (NOS) catalyze the oxidation of L-Arginine to nitric oxide molecule and L-citrulline.[2] Mammals contain three different NOS isozymes: neuronal NOS (nNOS, in the brain), inducible NOS (iNOS, in macrophage cells) and endothelial NOS (eNOS, endothelial cells of blood vessels).[3] Indeed, NO is a free radical gaseous molecule under normal conditions it is highly toxic substance to healthy cells. In our body, it is produced locally at proper concentrations at proper times.[4] In endothelial cells it relaxes smooth muscles causing a decrease in blood pressure. Macrophage cells generate NO as an immune defense system to destroy microorganisms and pathogens.[5, 6] The overproduction of NO, especially by nNOS (in brain) is implicated in various disease states such as neurodegeneration, oxidative stress, stroke, migraine and chronic headache, Parkinson,[7] Alzheimer,[8] and Huntington diseases,[9] tissue damage, hypotensive crises during septic shock, arthritis, and various kinds of inflammatory diseases.[10, 11] It also reacts with other free radicals to create specific molecular modifications. For this reason, it is important to inhibit selectively nNOS isoform in the brain without inhibiting the eNOS and iNOS isoforms.[12] However, it is a very difficult task to design a selective nNOS inhibitor because of the high active site similarities (**Scheme 1, Figure 1 and Table 1**) of the NOS isoforms. In the literature there are many outstanding studies, however, no drug, which accomplished the required potency and selectivity has yet been developed and clinically trialed.[13] Therefore, developing a clinical agent that selectively inhibits nNOS, to decrease the excess generation of NO production in the brain remains a challenging task.

In this study, *in silico* modeling methods were used to design inhibitors with high affinity and selectivity

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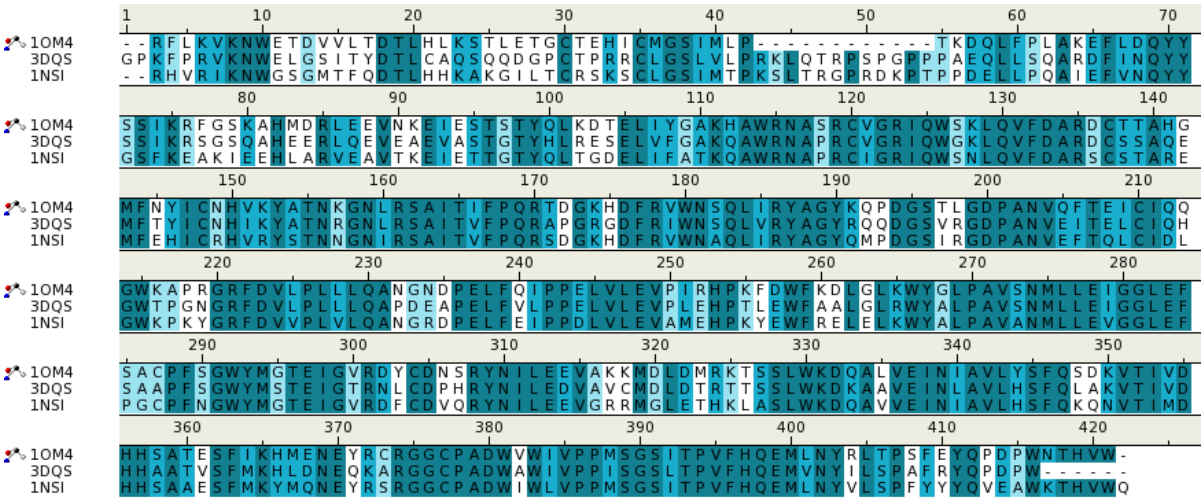


Scheme 1 Alignment of the residues of the three NOS isoforms. The iris blue color shows exact matching, light blue color partial matching and white color unmatching residues.

Figure 1 Superposition of the backbone of the three NOS isoforms (The colors designate; 1OM4: yellow, 3DQS:green, 1NSI: magenta)

Table 1. Some active site residues of NOS isoforms: the differences are indicated in bold.

Residue numbers in aligned isoforms	Residue numbers in original crystal structures		
	nNOS	eNOS	iNOS
289	F584	F355	F369
290	S585	S356	**N370**
292	W587	W358	W372
293	Y588	Y359	Y373
297	E592	E363	E377
301	R596	R367	R 381
302	D597	**N368**	D382
305	D600	D371	D385
383	W678	W449	W463
411	Y706	Y477	Y491

toward nNOS isoform based on the known target binding sites of three isoforms. Several hundred compounds were screened *in silico* for prioritization of lead candidates. *De novo* design method was used rationally for the modifications and additions to the selected scaffold within a target-binding site in order to enhance its binding affinity and selectivity to that isoform. [14] The best candidates showing high affinity and selectivity against nNOS over eNOS and iNOS isoforms were determined.

2. Result and Discussion

Numerous computational modeling and re-docking studies were carried out with the aim of obtaining additional validation and support for the computational results obtained for NOS isoforms. The ligands, which were crystallized together with their isoforms, were taken out, redrawn, minimized and re-docked into their isoforms. The root mean square deviations (RMSD) of the docked poses of the ligands were compared to the original co-crystalized position of the ligands within the active sites of the isoforms. The docked and co-crystallized ligands' poses overlapped and generated acceptable (1.9 – 2.2 Å) RMSD values.[15]

This study was based on the scaffold (**Figure 2**) which was selected from more than several hundred lead compounds in the ZINCv12 lead library for their structural and physicochemical properties which selectively inhibit nNOS isoform.[16] Utilizing ZINC and Accelrys 3.1 fragment-based libraries, which contain about four hundred thousand fragments, fifty potential candidates were selected out of a few hundred thousand fragments based on scoring values in the active site of the nNOS isoform using Accelrys's de Novo Design module. All these new scaffold analogues were docked into the active sites of nNOS, eNOS and iNOS isoforms. Only nNOS isoform selective ones were used for further modification. The structure-based methods were employed manually for the further optimization of the potential nNOS inhibitors by adding and removing a few fragments on the inhibitors. Our current design and computational evaluation of ten potential nNOS selective inhibitors using various docking tools are listed in **Scheme 2.**

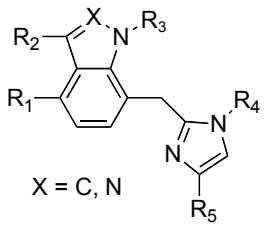
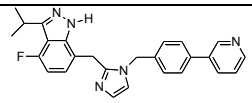
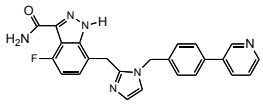
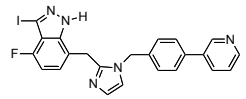
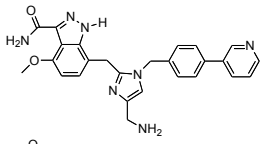
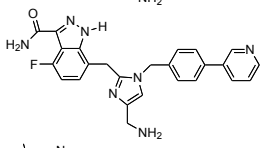
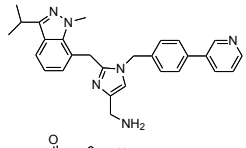
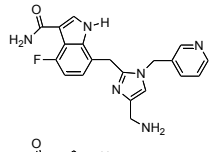
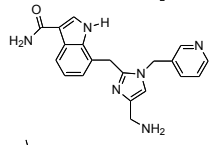
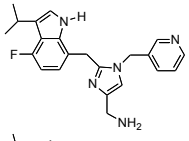
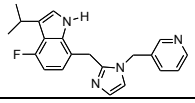


Figure 2. *Lead scaffold used in this study*

Code	Chemical Structures	Estimated Ki (nM)		
		nNOS (1OM4)	eNOS (3DQS)	iNOS (1NSI)
1		96	1180	218
2		176	389	491
3		80	239	176
4		82	444	176
5		89	104	330
6		176	755	432
7		42	943	689
8		35	2630	145
9		100	1410	189
10		1460	790	1150

To render visible the detailed interactions of the docked poses of the designed inhibitors, compound **7** was selected for the three isoforms. Analysis of the optimal binding mode for compound **7** (**Figure 3 A and A-1**) in the nNOS active site cavity revealed that this compound is located in the vicinity of the heme cofactor. Compound **7** interacts with active site residues lining the cavity as well as with the heme cofactor. Two pi-cation interaction forms between the amino cation (-$^{+}NH_3$) of the side chain of ARG603 and the indane ring of the inhibitor. The hydrogen atoms on the methyl amino group of inhibitor **7** make three strong H-bonds (distances are 1.774 Å and 2.171 Å) with two propionate side chains of the heme cofactor. The nitrogen atom of the pyridyl group makes another strong H-bond with one of the H-N moieties of the heme group as seen in **Figure 3 A-1**. The amide hydrogen atoms of inhibitor **7** make bifurcated interactions with the ASP600 side chain carboxylate group with a distance of distances are 2.237 Å (**Figure 3 A**). In addition to these bonds fluoride atom makes another close interaction with the side chain of ARG596. The binding mode adopted by compound **7** fits snugly within a cavity lined with active site amino acid residues. This pocket includes VAL565, GLN478, PRO565, ARG481, GLU592, TRP678, ARG596, ARG603 and ASP600. The side chains of these residues are in contact with compound **7** with either polar or hydrophobic interactions. **Figure 3 B** and **B-1** show the various interactions between inhibitor **7** and eNOS isoform. The GLU377 side chain interacts strongly with the 1-aminomethyl H- atoms of inhibitor **7** as in **Figure 3 B-1**. The hydrogen atom of the imidazole group of the inhibitor makes a H-bond with heme. The side chain of the ASN354 makes another important H-bond with the amide carbonyl group of inhibitor **7**. It is interesting to see that within the vicinity of the 3.5 Å distance not as many active site cavity amino acid side chains interact with the inhibitor **7** as in nNOS isoform. Having the lesser of number of H-bonds and fewer number of interacting side chains make this inhibitor weaker than that of nNOS isoform. The other interaction residues are VAL352, THR121, PRO350, GLN263, ASP381, TRP463 and TYR491. **Figure 3 C and C-1** showing compound **7** within the active site of iNOS isoform. The NH hydrogen atom of the benzopyrrole group of compound **7** makes a H-bond with the propionate oxygen of the heme cofactor. The amide carbonyl of the benzopyrrole group of the inhibitor makes another H-bond with the hydrogen atom of the ASN340 side chain. The 1-aminomethyl group of the imidazole ring of the inhibitor makes the three important H-bonds; first one with the GLU377 side chain oxygen and the two more with heme group. The other interacting active site residues of iNOS isoform are ASN338, TYR359, ALA337, GLY357, PRO336, and VAL338. In this study the best ten selected nNOS isoform inhibitors generally bind tighter to iNOS and eNOS. For compound **8** eNOS selectivity ratio (eNOS/nNOS) is 75.14, whereas iNOS selectivity ratio (iNOS/nNOS) is only 4.14. The highest selectivity of nNOS over eNOS and iNOS was obtained as 22.45 (eNOS/nNOS) and 16. 40 (iNOS/nNOS) for compound **7** in this series. In compound **10** removing 1-methyl amine moiety from imidazole ring totally abolished both nNOS selectivity and potency. Another important finding within this series is that the removing of the benzene ring between the imidazole and pyridyl ring increased both nNOS selectivity and inhibition potency. Substitution of the benzopyrazole ring with benzopyrrole ring also increased nNOS selectivity and potency. All these modifications decreased molecular weight by increasing druggability properties of the designed compounds. It was found that the positions 4 and 6 of the benzopyrrole ring and position 1 and 4 of the imidazole ring of this scaffold are vital for selectivity and potency for nNOS isoform.

3. Experimental

Materials and methods: Discovery studio 3.1 (Accelerys), Autodock 4.2 and MGL Tools 5.6.1 were used for running the simulation. The simulation studies are carried out in DELL Precision T3600 with Intel Quad core processor and 8GB RAM installed with Linux OS.

3.1. Ligand and Enzyme Preparations

The crystal structures of nitric oxide synthases were retrieved from RSCB protein data bank (pdb) (http://www.rcsb.org) for computational studies. Among all NOS PDB structures, 1OM4[17] for nNOS, 3DQS[18] for eNOS and 1NSI[19] for iNOS isoforms were

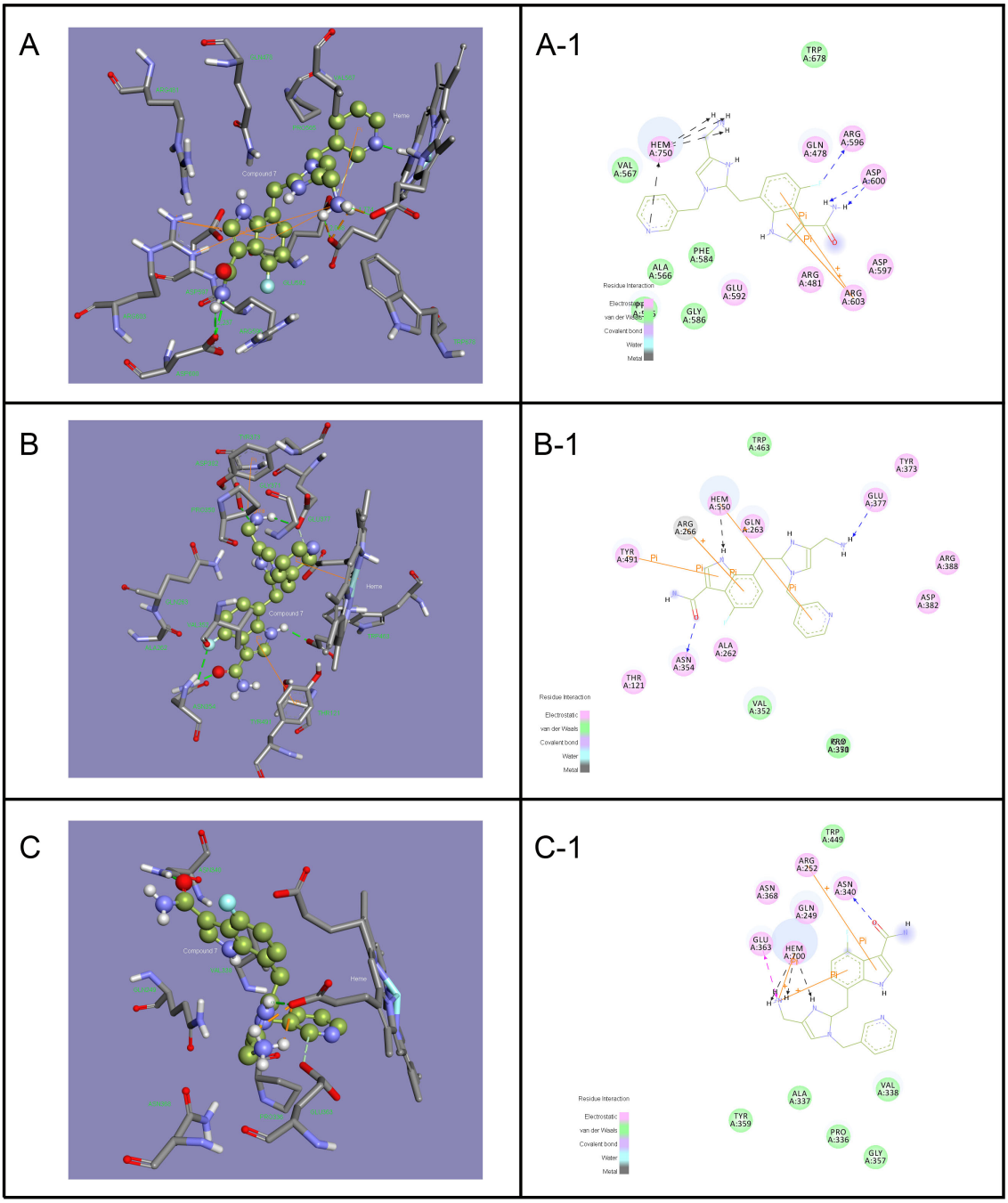


Figure 3. The predicted 3-dimensional orientation of compound **7** in the active site of the nNOS (A), eNOS (B), and iNOS (C) isoforms are given. Amino acid side chains within the volume of 3.5Å distance from the inhibitor are shown as sticks, the inhibitor is shown as a ball and stick, the cofactor HEME is depicted as sticks line. Dashed green lines indicate either electrostatic or hydrogen bond interactions.

chosen. The validation of the *in silico* docking method was performed by re-docking of the bound inhibitors of the experimental X-ray structures and comparing the poses of the lowest energy conformation predicted by the scoring function with the experimental poses. In general, binding poses generated by docking method are very close to experimental orientations with root mean square deviation (RMSD) values of 1.9 – 2.2 Å.

Discovery Studio 3.1 was employed to minimize and prepare enzymes and ligands for docking experiments. For enzyme preparation, except for the oxygenase domain of chain A, all non-interacting ions and molecules, chains and all solvent molecules existing in PDB structures were removed. However, the heme group, zinc ion and tetrahydrobiopterin (H_4B) were kept in the active site.

Using a fast Dreiding-like force field, each protein's geometry was first optimized and then submitted to the "Clean Geometry" toolkit of Discovery Studio (Accelerys, Inc.) for a more thorough check. Missing hydrogen atoms were added based on the protonation state of the titratable residues at a pH of 7.4. Ionic strength was set to 0.145 and the dielectric constant was set to 10. The detailed procedure was described elsewhere.[20, 21]

3.2. Docking

Protein-ligand docking calculations were carried out using AutoDock 4.2 software.[22] AutoDock Tool (ADT)[23, 24] interface was performed for the preparation of, AutoGrid parameter files (gpf) and AutoDock parameter files (dpf). Beforehand, the charge of the Fe atom of the heme molecule in all three enzymes was assigned a charge of +3 and the Gasteiger-Marsili atomic charges were calculated for the other atoms. The ligands were docked inside a grid box with 70 Å x 70 Å x 70 Å dimensions with grid spacing of 0.375 Å. For the center of the grid box, centers of ligands found in original PDB structures' active site were noted and used for all docking simulations.

The Lamarckian genetic algorithm (LGA) was selected and parameters were set to 10 in independent LGA runs, 150 in population size, 5000000 in energy evaluations and 27000 in generations and default settings were used for all parameters. Full flexibility was applied for all docked ligands while keeping the isoforms rigid at their minimum conformational states. Inhibition binding constants (K_i) of best runs in each resulting docking orientations were collected and the relevant tools of Accelrys Discovery Studio 3.1 were used to analyze the poses and interactions.

Conclusion

The active sites of three NOS isoforms show great similarity; therefore, designing of selective nNOS inhibitors is a very difficult task. The computational results carried out with all of the docking tools clearly demonstrate that the selected scaffold is a potential candidate for further modifications and optimization for designing selective and potent nNOS inhibitors. Subtle differences in the conformations of amino acid sequences (e.g. ASP597 in nNOS) of the three isoforms in the active site regions, as discussed above, were the determining factors for the selectivity and the potency of the compounds. The potency and the selectivity of nNOS isoform were achieved by introducing 1-methyl amino group at the forth position of the imidazole moiety of the best inhibitor **7**. The positively charged 1-methyl amino group makes three H-bonds with the two-propionate groups of the heme cofactor, which does not occur in eNOS and iNOS. Removing of 1-methyl amino group from compound **10** totally abolished both potency and selectivity for nNOS. Newly designed inhibitor **7** shows nNOS inhibition 23 and 17-fold better than that of eNOS and iNOS, respectively. The computer-aided drug design of novel drug candidates for nNOS isoform, as reported in this study, will be a starting point for the synthesis of new, potent and selective nNOS inhibitors the future research studies.

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