

KADIR HAS UNIVERSITY
GRADUATE SCHOOL OF SCIENCE AND ENGINEERING



IN SILICO DESIGN OF SELECTIVE NEURONAL NITRIC OXIDE
SYNTHASE INHIBITORS IN ORDER TO PREVENT
NEURODEGENERATIVE DISEASES

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ABSTRACT

Nitric Oxide synthases (NOS) are the family of enzymes which catalyzes the oxidation L-Arginine amino acid to nitric oxide molecule (NO) L-citrulline. Mammals contain three different NOS isozymes: Neuronal NOS (nNOS, in the brain), inducible NOS (iNOS, in macrophage cells), endothelial NOS (eNOS, the inner walls of blood vessels).

Nitric Oxide (NO) is an important messenger molecule, which regulates several physiological functions in cardiovascular system and neuronal cells in the brain.

Indeed, NO is a free radical gaseous molecule under normal conditions and highly toxic substance to our cells. In our body, it is produced locally at proper concentration at proper time. In endothelial cells, it relaxes smooth muscle causing to decrease blood pressure. Macrophage cells generate NO as an immune defense system to destroy microorganisms and pathogens.

In our brain under certain pathological conditions after a certain ages produced excessive NO, causes tissue damage and oxidative stress. This leads are a variety of neurodegenerative diseases, such as Alzheimer's disease, rheumatoid arthritis and Parkinson's diseases. For this reason, it is important to inhibit selectively neuronal isozymes of NOS, nNOS in the brain. Three isozymes show extraordinarily structure similarities hindering the selective inhibitor design. In the literature there are many outstanding studies, however there has not being developed any drug which

accomplished the required affinity and selectivity. Neurodegenerative diseases were very common death cause after cardiovascular diseases and cancer in the developed countries. We plan to use computer modeling based on the known crystal structure of three NO isozymes. In this project computationally, we developed, highly selective nNOS inhibitors via *in silico* screening. The inhibitors whose experimental inhibition values reported up to now were tested within our prepared model NOS isozymes. The obtained computational binding constants were compared with literature experimental values. The enzymes whose experimental values agreed with computational values were chosen for further studies. First, several suitable scaffolds (leads) were determined from lead library of ZINC database. These leads were optimized using fragment library of ZINC and Accelrys in the nNOS active site. The new selective and potent inhibitors were determined as a result of *in silico* screening. The inhibitors binding energy and inhibition constants toward nNOS, eNOS and iNOS enzymes are reported. In the future project, a collaborative work is going to be searched for the synthesis and enzymatic work of these determined inhibitors.

Key words: Nitric oxide synthase, nNOS, eNOS, iNOS, docking, scoring, molecular modeling, *in silico* screening

Özet

Nitrik oksit sentazlar (NOS) L-Arginin aminoasidini L-sitrüline oksitleyerek Nitrik oksit molekülü üreten bir enzim ailesidir. Memelilerde NOS enziminin nöronal NOS (nNOS, beyinde ve omurilikte), indüklenebilir NOS (iNOS, makrofajlarda), endotelial NOS (eNOS, kan damarlarımızın iç çeperlerinde) olmak üzere üç izoenzimi bulunur. Bu üç değişik izoenzimden üretilen Nitrik oksit (NO) kardiovasküler sistemden, bağışıklık sistemindeki fizyolojik fonksiyonları da içeren bir dizi görevi üstlenen bir sinyal molekülüdür. Aslında NO normal şartlarda gaz halinde, radikal ve çok toksik bir moleküldür. Bünyemizde uygun zamanda uygun derişimde üretilir ve lokal olarak kullanılır. Beyinde nörotransmitter görevi üslenen NO ileri yaşlarda kontrolsüz üretildiğinde doku hasarına ve oksidatif strese sebep olur. Bu durum da Alzheimer, romatoid artrit ve Parkinson gibi nörodejeneratif hastalıklarının oluşumuna katkıda bulunur. Damar iç çeperlerinde endotelial tabakada üretilen NO damara esneklik vererek kan basıncının düşmesine neden olur. Vücudumuza giren mikroplara karşı bizi koruyan makrofajlarda NO molekülünü silah olarak kullanır. Dolayısı ile sadece beyinde üretilen NO molekülünün azaltılması için nNOS enziminin

seçimli olarak inhibe edilmesi çok önemlidir. Üç izoenzimin yapısı olağanüstü bir şekilde birbirlerine benzemektedir ve bu benzerlik nNOS seçimli inhibitör tasarımını zora sokmaktadır. Birçok çalışma olmasına rağmen henüz bunu başaracak bir ilaç geliştirilememiştir. Nörodejeneratif hastalıklar, gelişmiş ülkelerde kalp damar hastalıkları ve kanserden sonraki en sık ölüm nedenidir. Bu projede amacımıza uygun olarak nNOS'a seçimli bir dizi inhibitör tasarlanmıştır. Bunun için hedeflenen izoenzimlerin yapılarını temel alan bilgisayar destekli ilaç tasarımından yararlanılmıştır. İlk olarak bugüne kadar çalışılmış önemli NOS hedefli inhibitörler sistemimizde her 3 izoenzim için test edilip benzerlik ve farklılıklar bir model oluşturulmak amacıyla incelenmiştir. Deneysel ve hesapsal sonuçları en çok destekleyen 3 izoenzimin (nNOS, eNOS ve iNOS) kristal yapıları *in silico* tarama yapılmak için seçilmiştir. Tespit edilen bu enzimlere karşı ZINC veri bankası taranarak uygun ana yapılar (leads), daha sonra da *de novo* programı kullanılarak ana iskeletlere fragmentler (ZINC ve Accelrys fragment kütüphanesi) eklenerek nNOS seçimli olan inhibitörler tespit edilmiştir. Çalışmanın sonucunda nNOS enziminin aktif bağlanma bölgesine seçimli olarak yüksek ilgiyle bağlanan inhibitörlerin yapıları, bağlanma enerjileri ve inhibisyon katsayıları verilmiştir. Bu sonuç raporu kapsamında ve ümit vaat edenler ileriki projelerde bu konuda çok ileri düzeyde araştırmalar yapan laboratuvarlar ile işbirliğine gidilerek denemeleri sağlanacaktır.

Anahtar Kelimeler: Nitrik Oksit sentaz, nNOS, eNOS, iNOS, docking, skorlama, moleküler modelleme, *in silico* tarama.

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1 INTRODUCTION

Considering the examples of national economies in the world, pharmaceutical industry and the R&D activities are becoming important increasingly. In addition to the high cost of research and development activities, laborious research-based pharmaceutical companies have increased their risks. At the end of the R&D processes enduring 12-14 years on average. (Silverman R. B., 2009) Thousand of molecules and time consuming development process are needed for a drug to place into a shelf on the pharmacy. Intelligent drug design is method to accelerate the finding procedure of new molecules. Determination of chemical drug candidates by screening and searching in silico environment gives direction to experimental studies.

Due to the potential benefit of treatment of neurodegenerative diseases, many pharmaceutical companies started programs to identify the selective nNOS compounds by the early 1990's . (Silverman R. B., 2009) NOS enzyme has three isoenzymic forms. Mammals contain three different NOS isozymes: Neuronal NOS (nNOS, in the brain), inducible NOS (iNOS, in macrophage cells), endothelial NOS (eNOS, the inner walls of blood vessels). eNOS in endothelial cells produce NO to regulate blood pressure. nNOS in neuronal cells produces NO for neurotransmission. iNOS in macrophage cells produces nitric oxide to combat infection and microorganisms stimulated by pathogens. (Silverman R. B., 2009) Under the presence of cofactors nicotinamide adenine dinucleotide phosphate hydrogen (NADPH), flavin adenine dinucleotide (FAD), flavin

mononucleotide (FMN) and tetrahydrobiopterin(BH4) NOS enzyme produces NO by oxidation of L-arginin terminal guadino group. (Silverman R. B., 2009) Nitric Oxide synthases (NOS) are the family of enzymes which catalyzes the oxidation L-Arginine amino acid to nitric oxide molecule (NO) L-citrulline. Nitric Oxide (NO) is an important messenger molecule, which regulates several physiological function in cardiovascular system and neuronal cells in the brain. (Silverman R. B., 2009)The overproduction of NO by nNOS or iNOS and the underproduction by eNOS have been shown to lead to pathophysiological conditions such as neurodegenerative diseases Alzheimer, and Parkinson diseases, stroke, rheumatoid arthritis, hypertension and atherosclerosis. Inhibition of nNOS can be therapeutic benefit. Five cofactors are required for the catalyses by NOS izoenzymes. (Silverman R. B., 2009) NADPH is in reductase domain and transfers two electrons to FAD, FMN, transfers one electron to heme in oxygenase domain. BH4 in oxygenase domain facilitates catalysis from L-arginin to L-sitruiline (Abu-Soud, Presta, Mayer, & Stuehr, 1997).

The expected lacking effects of Nitric Oxide enzymes are observed on the transgenic mice for all NOS isoenzymes (knock-out). (Silverman R. B., 2009) As a result of these experiments, without the hypertensive effect of inhibition of eNOS or without causing immune problem for iNOS inhibition, nNOS inhibitor will have protective effect on neuro-degenerative diseases. Due to the potential benefit of treatment of neurodegenerative diseases, many pharmaceutical companies identify compounds by the selective nNOS programs initiated in the early 1990s. Since the crystal structure could

not obtained previously, the general approach is using of L-arginine as a substrate for the lead and applying structural changes on it with the hope of selective binding analogues to nNOS, eNOS and iNOS. (Silverman et al. 2009). As a result of the lack of selectivity studies, these three enzyme substrate, an active region and the reaction are very similar. Any modifications on L-arginine in the active sites will have similar effects. A few designed compounds bind like an anchor to active site, try to reach out second cavity of aminoacids (Silverman et al. 2003).

In this thesis we aimed to determine the potential inhibitors based on the eNOS, iNOS, nNOS enzymes structures but only which is selective for nNOS enzymes. An inhibitor that prevents neuronal degenerative formations which is caused by accumulation of excess nitric oxide in brain cells in elderly ages contributing to Alzheimer treatment research will be designed. Autodock protocols, Accelrys Discovery Studio, Lipdock protocols that have computer aided drug design methods are utilized based on the crystal structures of NOS isozymes. The most important reported inhibitors up to now are tested with our prepared NOS isozymes and their obtained computational binding constants are compared with literature experimental values. From these lead scaffolds new, more selective and potent inhibitors are designed. The results of this study, tens of thousands derivatives scanned with experimental high-throughput screening method (HTS) and identified for potential drug molecules, so time and money will be saved.

1.1 Background of Nitric Oxide Synthase

Nitric Oxide synthases (NOS) are the family of enzymes which catalyzes the oxidation of L-Arginine amino acid to nitric oxide molecule (NO) and L-citrulline. (Silverman, Roman, Martasek, Gomez-Vidal, & Ji, 2006) Mammals contain three different NOS isozymes: Neuronal NOS (nNOS, in the brain), Inducible NOS (iNOS, in macrophage cells), Endothelial NOS (eNOS, the inner walls of blood vessels). Nitric Oxide (NO) is an important messenger molecule, which regulates several physiological functions in the cardiovascular system and neuronal cells in the brain. (Silverman, Roman, Martasek, Gomez-Vidal, & Ji, 2006) Different isoforms of NOS have different cell and tissue structures. Catalytic center of nitric-oxide synthase is composed of two domains. The C-terminal domain is reductase domain that binds FMN, FAD and NADPH. The N-terminal oxygenase domain contains heme, H₄B and the substrate L-Arginine. (Abu-Soud, Presta, Mayer, & Stuehr, 1997) The oxygenase domain of the three NOS isoforms is almost identical. (Abu-Soud, Presta, Mayer, & Stuehr, 1997) Five cofactors are necessary for catalyzed by NOS. Reductase domain can transfer the electron to the oxygenase domain for enzymatic activity. Reductase domain and oxygenase domain are linked by binding of a calcium-calmodulin motif. (After & Feldman, 1993) Electrons flow from NADPH through FMN and FAD in one subunit to the other subunit. (Silverman R. B., 2009). There are three known NOS isoforms. Two are constitutive form and the third one is inducible form. The gene coding for iNOS is located on

chromosome 17 and for nNOS is located on chromosome 12. Gene coding for eNOS is located on chromosome 7. Three isozymes show extraordinarily structure similarities for preventing the selective inhibitor design. For this reason, it is important to inhibit neuronal isozymes of NOS in the brain selectively. (Silverman,, et al., 2008)

1.2 eNOS

eNOS controls the smooth muscle tone primarily. It relaxes smooth muscle so blood pressure is decreased in endothelial cells. (Hah, Martasek, Roman, & Silverman, 2003)The crystal structure for the heme domain of eNOS has been solved. Zinc ion is coordinated tetrahedral by two pairs of cysteine residues and is located at the eNOS dimer interface. Enos is a dimer that contains two similar monomers and it should have dimeric form to be functional. The gene that codes monomers is on the 7q35-36 chromosome and contains 26 ekson. Firstly heme molecule links the enzymes and then enzymes take dimeric form. The absence of the heme group, enzymes remains monomer. When the enzyme goes to be dimeric form, BH₄ come up to link and the dimer becomes stable. Then Zn ions are linked to the stable form of enzymes. (Erdal, Litzhger, Seo, Zuhu, Ji, & Silverman, 2005) (Poulos, Silverman, Jamal, Li, Xue, & Delker, 2010) Functional activity of the dimer depends on the number of connected BH₄. Not connection of the BH₄ can produce the dimer form of O₂ and connection of the one BH₄ molecule; both O₂ and NO are produced by eNOS dimer. If the presence of high level of BH₄ creates

dimer enzymes, NO are created. The location of the enzyme in the cell is un clear. Enos is located depending on the cell membrane in the cell. (Arnet, McMillan, Dinerman, Ballermann, & Lowenstein, 1996)

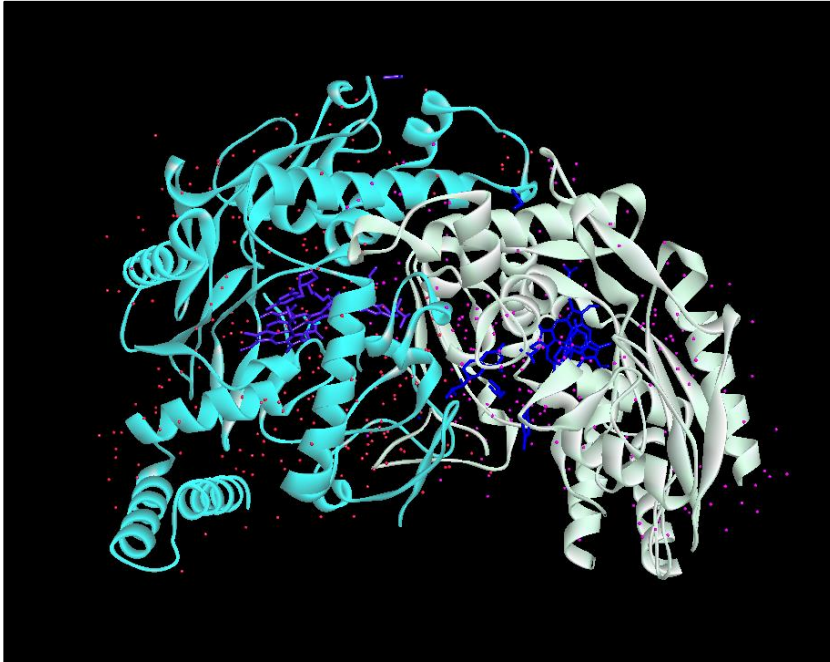


Figure 1 Original three-dimensional appearance of the eNOS enzyme



*Figure 2 Original three-dimensional view of the eNOS enzyme has been minimized.
(Single domain)*

1.3 iNOS

NO is generated by macrophage cells as an immune defense system to demolish microorganisms and pathogens. Large amounts of NO in the immune defense system are produced by the inducible isoforms iNOS. (Silverman R. B., 2009)

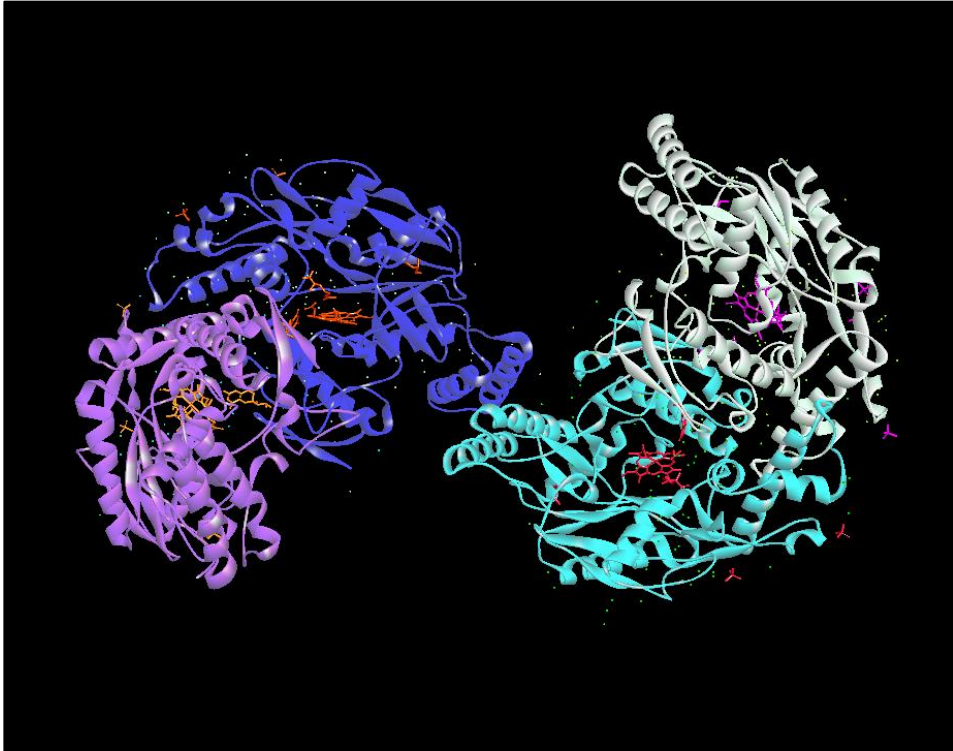


Figure 3 Original three-dimensional appearance of the iNOS enzyme



*Figure 4 Original three-dimensional view of the iNOS enzyme has been minimized.
(Single domain)*

1.4 nNOS

The neuronal nitric oxide synthesis that is involved in the development of nervous system products such as NO in neuronal cells. It regulates the release of neurotransmitters so synaptic plasticity, memory function and neuroendocrine secretion are controlled by NO. It is significant for memory and learning. (Erdal, Litzhger, Seo, Zuhu, Ji, & Silverman, 2005) Neuronal nos is related plasma membranes so nNOS carry out a role in cell communication. NOS1 gene encodes nNOS in chromosomal region 12q24 and this gene contains large amount polymorphic repeats of eNOS and nNOS.

nNOS are located in cytosol. (Ji, Li, Flinspach, Poulos, & Silverman, 2003)

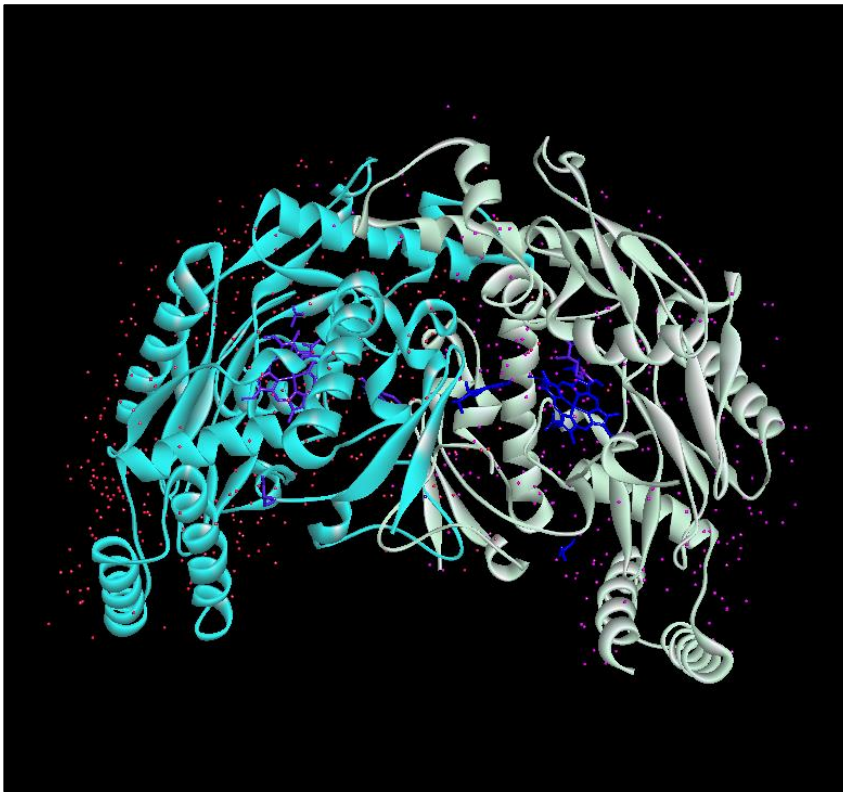


Figure 5 Original three-dimensional appearance of the nNOS enzyme



*Figure 6 Original three-dimensional view of the nNOS enzyme has been minimized.
(Single domain)*

1.5 Nitric-oxide

Nitric-oxide is a free radical molecule in both intracellular and extracellular structure form. Half-life of NO is 2-5 seconds. Many cellular processes are regulated by the release of NO from NO-producing cells. In addition NO is a neurotransmitter that pass freely through the cell membrane. (Silverman R. B., 2009) NO is a free radical gaseous molecule under normal conditions and highly toxic substance to our cells. In our body, it

is produced locally at proper concentration at proper time. If its production and consumption go wrong, then serious pathological and physiological diseases will arise. In neuronal cells, NO regulates the release of neurotransmitters and is involved in synaptic plasticity, memory function and neuroendocrine secretion. In endothelial cells, it relaxes smooth muscle causing to decrease blood pressure.

Macrophage cells generate NO as an immune defense system to destroy microorganisms and pathogens. In our brain, under certain pathological conditions after certain ages produced excessive NO, causes tissue damage and oxidative stress. (Silverman, Roman, Martasek, Gomez-Vidal, & Ji, 2006)

The overproduction of NO by nNOS or iNOS and the underproduction by eNOS have been shown to lead to pathophysiological conditions such as neurodegenerative diseases Alzheimer, and Parkinson diseases, stroke, rheumatoid arthritis, hypertension and atherosclerosis. Inhibition of nNOS can thus be of considerable therapeutic benefit. However inhibition must be isoforms selective so that only NO formation by the disease-associated NOS will be inhibited by the treatment, while the physiological function of the other isoforms often eNOS is unaffected. Therefore, it is important to inhibit selectively neuronal isozymes of NOS, nNOS in the brain. (Silverman., et al., 2008)

1.6 Mechanism of NO Production

No production are took place in two identical steps by all three NOS isoforms. In the first step, L-Arg is transformed Ng-hydroxy-L-Arg (NHA) at the heme active site of NOS enzymes. In the second step, NHA is oxidized to NO and citrulline. (Silverman R. B., 2009) For catalysis reducing enquired are transferred from NADPH through FAD to FMN. Finally they transfer to the heme group. NADPH is in reductase domain and transfers two electrons to FAD, FMN, and transfers one electron to hem in oxygenase domain. BH4 in oxygenase domain facilitates catalysis from L-margining to L-citrulline (Silverman,, et al., 2008)

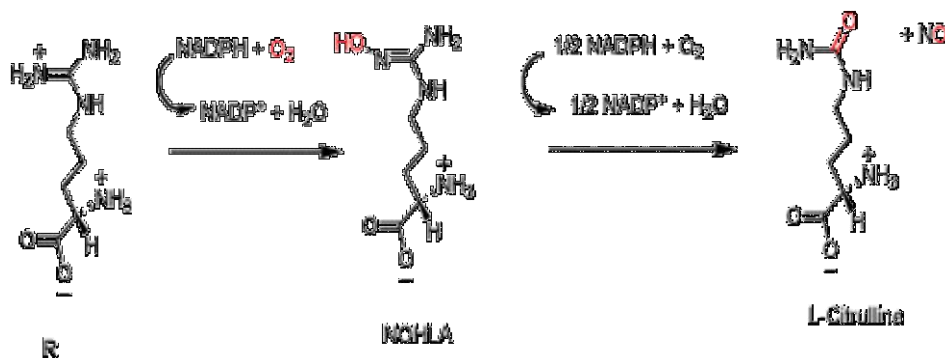


Figure 7 Nitric Oxide Production from L-Arginine by NOS enzyme

1.7 The Substrate L-ARG of NOS

The original substrate L-Arg of Nos binds directly above the heme iron (Fe) atom.

Another cofactor H4B binds along the side of the heme. Moreover, the substrate L-Arg and H4B are joined together through an extended H-bonding mediated network by one of the two propionate groups of the heme. (Zhang, Fast, Marletta, Martasek, & Silverman, 1997)

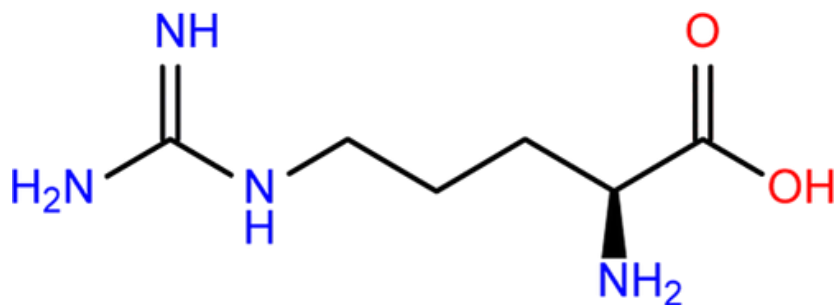


Figure 8: Chemical structure of L-Arginine

1.8 Active site and CO-Factors of NOS

1.8.1 H₄B

H₄B is a cofactor of all three nos enzymes isoforms that is essential for the catalytic .Biochemical studies showed that H₄B play a role in protein stability, monomer-dimer equilibrium, proteolytic susceptibility, heme-ligand binding and substrate binding properties. (Shearer, et al., 1998) A cellular level is more important for structure of NOS enzymes. H₄B binding make happen to increases L-arginine binding and to shift heme iron of NOS to high-spin state so in NOS enzymes stabilizes the active form of enzymes. Generation of NO is occurred by suboptimal concentration of H₄B. (Silverman R. B., 2009)Another possible role of H₄B is to perform a one electron donor to the heme iron for cleavage of the o-o bond.

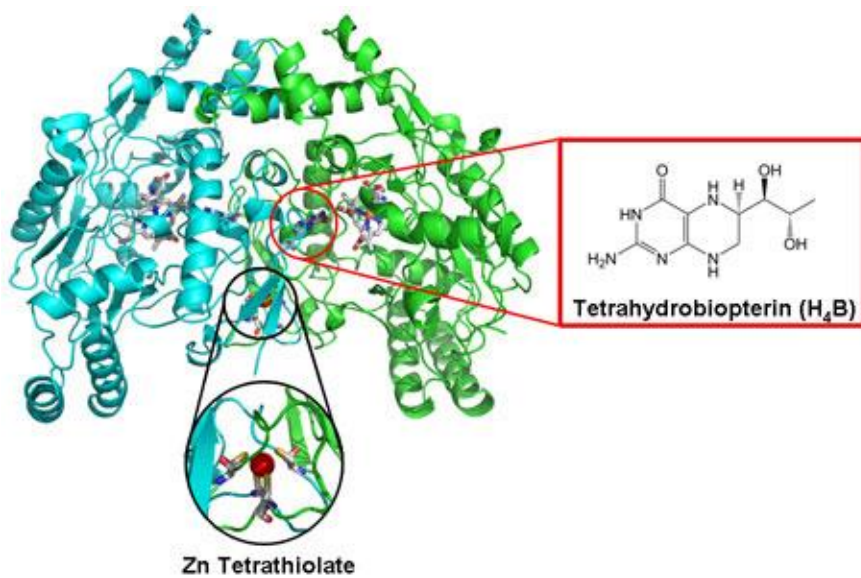


Figure 9 H4B layout in nos enzymes in stick representation

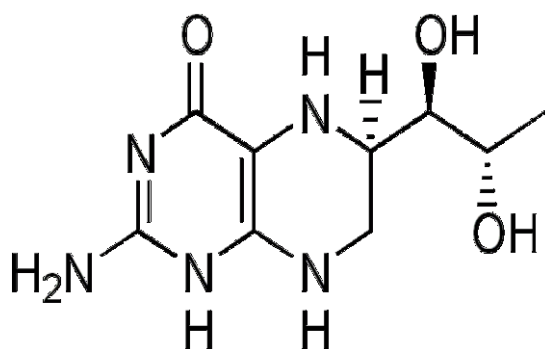


Figure 10 Chemical structure of tetrahydrobiopterin

1.8.2 Zn

Zn plays a role such as cofactor in the catalytic activity. In the dimer interface of NOS enzymes Zn-tetrathiolate center helps dimer stabilization. It creates formation of the pterin binding site. (Silverman R. B., 2009)

1.8.3 The Heme Iron

The iron of heme takes a place in the porphyrin ring. It is coordinated by pyrole nitrogen atoms of the porphyrin ring. A study shows that Fe-S bond is important for the catalytic activity of all NOS. Only Fe⁺²-diatomic ligand complex cause to be stable the NO complex. On the heme iron the decrease of positive charge in being reduced from Fe⁺³ to Fe⁺² allows to move closer to the heme iron for more desirable no bounded contacts. (Li, Raman, Martasek, Masters, & Poulos, 2001)

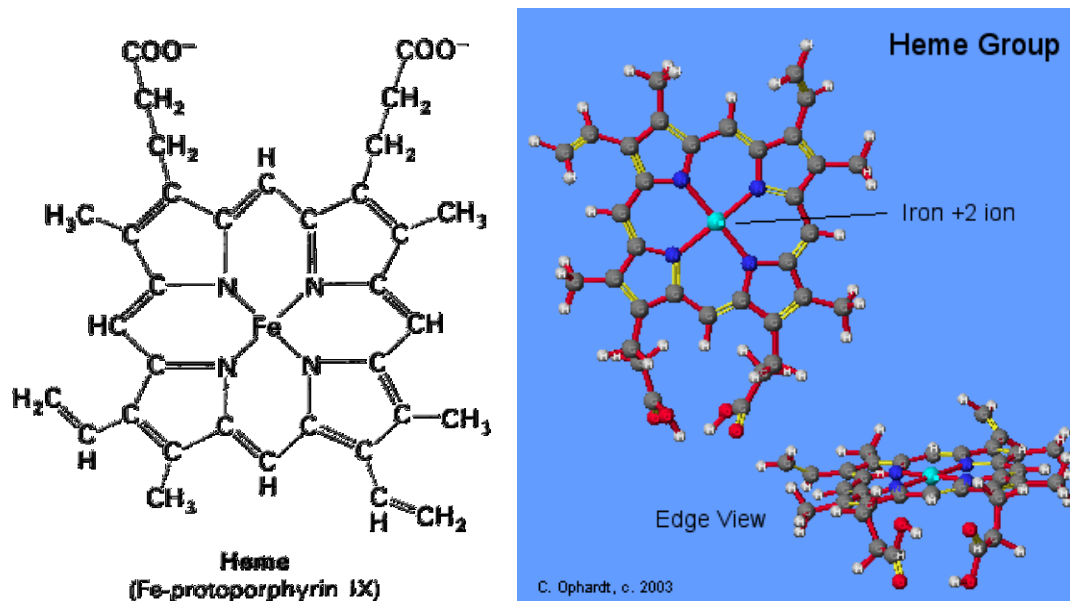


Figure 11 Shows that iron of the heme and Fe-S bound in stick representation

Computational Methodology

1.9 General Approach to the Molecular Docking

Molecular docking is the most important and useful key tool in computational biology.

Molecular docking studies are used to define the interaction of two molecular structures.

These molecules are small molecules known as ligand. In this context large molecules

known as a protein. It finds the best orientation of ligand in receptor active site. When

ligand come in contact any residue of receptor molecules, this protein cavity become receptor active site so a protein cavity where binding interaction come true, is described a receptor binding site by using the position of known ligands. The receptor binding site is most important for all docking. Molecular docking form is a complex form with minimum energy.

Aim of molecular docking is predicting molecular identification, conformation and determining optimal binding energy between two molecular structures.

For my thesis, molecular docking has main application in Drug discovery and designing. The result of molecular docking is analyzed by scoring function. Scoring function converts interacting energy that called as the docking score. Scoring function is mathematical methods for the predicting non_covalent interaction. It is called as binding affinity between ligand and target protein after they have been docked. Scoring functions have been improved for predicting intermolecular interactions,

Binding Energy:

$$\Delta G_{\text{bind}} = \Delta G_{\text{vdw}} + \Delta G_{\text{hbond}} + \Delta G_{\text{elect}} + \Delta G_{\text{conform}} + \Delta G_{\text{tor}} + \Delta G_{\text{sol}}$$

Docking score is numerical values so interaction energy is calculated. Then the most important state is analyzing the 3D pose of the bound ligand. It can be visualizing tools like Accelrys Discovery Studio. Modes of protein ligand interaction help to protein and ligand annotation. (DISCOVERY STUDIO 3.0,protocols)

There are majorly two type of docking. Flexible docking and rigid docking. For drug discovery and designing performed rigid docking. In rigid docking the target receptor protein and ligand is kept fixed during the molecular docking. This docking algorithm is based on geometric, volumetric and atomic coordinates of the target receptor protein. But sometimes some problems may occur. For example rigid body molecule as a target receptor cannot be a model all receptor molecules and in reality proteins are not stable in cells. Rigid-body docking occurs more quickly than the others. The flexible docking approach is harder than performing of rigid-body docking. (DISCOVERY STUDIO 3.0,protocols)

There are four major steps in molecular docking:

1. Preparing the Target Receptor: Firstly 3D structure of target protein should be downloaded from protein-data bank. This structure includes water molecules, non stabilize charge, missing residue. They should be completed according to parameters.

2. Determining of the Active Site: Active site where binding interaction come true should be identified. The target receptor protein has many active sites but of interest should be chosen.

3. Ligand Preparation: Ligands are in the various databases like ZINC, and literature. The Lipinski's Rule of 5 should be applied when the ligands are selected. These rules are very important for drug development. Lipinski' rule for the selecting ligand

- Not more than 5 –H bond donors.

- Molecular Weight not more than 500 Da.

- Log P not over 5 for octanol water partition coefficient.

- Not more than 10 H bond acceptors. (LIPINSKI@PFIZER.COM)

4. Docking: The last step, the ligand is docked in the target protein. Then interactions are checked. The ligand the best fit is selected.

1.10 Docking Tool : Auto Dock Version 4

AutoDock 4.0 is a docking tool that is open source to simulate the interactions between flexible ligands and target macromolecules. It was used genetic algorithm and grid-based method for energy evaluation. Standard procedure for docking produces per ten independent results for each ligand for docking flexible ligands and twist angles. AutoDock is contemplated to guess how ligands bind to a target protein of known 3D structures. Therefore, autodock is a crucial tool for drug design. (AutoDock Protocol)

1.10.1 Genetic Algorithm

Energy calculations in AutoDock are managed by producing grid maps before starting the docking. Grid maps are calculated by Auto grid and then created by placing the receptor protein inside that it is defined 3D grid box by using. Probe atom is located on each grid point by Auto grid. It divided grid spacing so grid maps are created for each type of created atom in docking. Grid maps are collected in separate grid files. Recalculation of the distances included in scoring function at each energy evaluation.

Genetic algorithms are used to search conformational space by ligand so slowest

energy conformation can be found (Autodock Tutorial).

AutoDock use Lamarckian genetic algorithm. The ligand is explained as a chromosome that has seen standard genes accounting for the ligand's cartesian coordinates. Torsional angles of ligands can also be identified. After that, genetic algorithm creates a population of random individuals with in grid box that also contain the target protein. Three translation(x, y, and z), four orientation and torsional angle genes are appointed randomly for each individual. These random individuals are converted into similar phenotype. (Autodock Tutorial)

AutoDock docking environment uses a semi-empirical force field based on the AMBER force fields. AutoDock uses a molecular mechanics model for enthalpic contributions. Van der Waals and hydrogen bonding is an empirical model for entropic changes. Each of these components is multiplied by obtained empirical weights from the calibration against a set of knowed experimental binding constants. The Lamarckian genetic algorithm of Autodock was used to research the conformational space. They are randomly assigned torsion angles to rotatable bonds and an overall rotation. 50 independent runs were carried out for each docking with each run that consist maximum 100 million energy evaluation. 300 distinct ligand conformers are initially generated and positioned randomly in the binding cavity. For all docking Lamarckian genetic algorithm was applied as well as all other docking parameters. Size of grid box was 70 Å in all dimensions. The distance between two grid points is set to 0.375 Å . (GRID, 2002)It is centered at the contained Fe atom in the center of Heme group. It covers the entire

binding site and its neighbour residues. Every docking consisted out of 100 GA runs and 10 independent runs. They are performed for each molecule. Maximum 50 million energy evaluations are allowed for each docking. All inhibition constants derived from binding energies that are calculated at the temperature 298.15 K.

Semi empirical free energy force field is used by Autodock 4.2 for estimation of ligand conformation. (AutoDock Protocol) Force field regards two parts. Ligand and target macromolecule are in the unbounded conformation.

At the beginning, intermolecular energy are calculated from conformation of ligand and target protein in unbounded state to bounded state. Secondly, intramolecular energetic of ligand-target receptor pair is estimated in their bound conformation. Free energy of binding equation is shown as below. The molecular energy calculated from total energy of hydrogen bond, desolvation, electrostatic energies, vander Waals, internal energy and unbound system energy.

1.11 Accelrys Discovery Studio

Accelrys Discovery Studio is a soft ware program. It provides molecular design solution for especially drug discovery area. Computational chemist and computational biologist

also use this program. It includes many modern methods for molecular docking and design new ligands. In my thesis Libdock, Cdock and Denovo protocols are used. For docking, enzymes are handled as a rigid. Ligands are handled as flexible. (DISCOVERY STUDIO 3.0,protocols)

1.11.1 Docking Tool: Ligand Docking with Libdock

Libdock is a docking tool that is hotspot matching for docking ligand molecules into an active receptor site. In other words it is fast, accurate docking method. At the beginning, a hotspot map is computed for the enzyme active site. There are two hot spot types: polar and a polar. Hotspot map is used to align the ligand conformation to form the best interactions in the enzymes. It uses enzyme site features to refer as Hotspots (K). After from favorable interactions, a final energy-minimization is calculated. Then the best scoring ligand poses are saved. First of all, before the docking, it is inevitable to bring out a receptor binding site so multiple conformations of ligands are into it. A binding sphere is necessary for libdock docking operation. (LibDock Protocol)

We used to cofactor's coordinate for creating the binding sphere and the default value of 100 for the number of hotspots. So the conformational time is increased. For binding site

image, a polar ligand atom such as a hydrogen bond donor or acceptor selects a polar hotspots and a polar atom such as a carbon atom selects an a polar hotspots. We also used the default value of 0.25 for the docking tolerance. RMSD tolerance (A0) contributes the matching algorithm to accept or reject any given match.

In Libdock Protocol, there are three catalyst algorithms for producing diverse ligand conformations. These conformational search procedures are Fast, Best and Caesar. Fast mode supplies low energy conformations. Best mode makes curtain more rigid energy minimization in cartesian space by using poling. Caesar is a very fast mode. This approach is also combined with consideration of local rotational symmetry. Conformation duplicates due to topological symmetry in a systematic search can be eliminated. (LibDock Protocol)

Libdock achieve docking in four different conformation methods

- High Quality

- High Search

- High Search for SASA (Solvent Accessible solvent Area)

- User specified

We also prefer fast search so we have most appropriate docking result and very large data set. (LibDock Protocol)

Libdock matching procedure, for example: A triplet of atoms A1, A2, A3 is considered a match to a triplet of hotspots, H1, H2, H3 so multiple conformations of the ligand generated. LibDock workflow starts conformational search like fast, caesar and then hotspots are generated related to target receptor protein. The next step is triplet match. Final optimization is the last procedure. A higher Libdock-score value indicates more favorable binding with small molecule and target protein. The ligands are sorted by the best LibDockScore for the best pose. (LibDock Protocol).

Libdock was preferred because of the following features:

- Doing features-based docking
- Fast speed
- It can be run in parallel
- It can generate catalyst conformers

1.11.2 Docking Tool: Ligand Docking with C-Dock

C-dock is a grid-based molecular docking method that applies CHARMM force fields. It uses molecular dynamics grid-based docking algorithm and find the random conformation in the target protein. While the small molecules are permitted to flex, the enzyme is held rigid. Small molecules like ligands are assured to approximately docked into the protein binding site. At high-temperature molecular dynamics are used to find possible ligand position in target rigid protein. Then ligand poses are evaluated with a grid representation in c-docker result. (Brooks, Bruccoleri, Olafson, States, Swaminathan, & Karplus,, 1983)

Firstly C-docker protocol improves pre-docked ligands using charmm. The binding receptor site sphere parameter can be bring out, if ligand is not pre-docked. It must be pointed out that before docking. Ligands must be into the binding receptor side. Several random ligand conformations have generated in the protein active site and procedure goes on heating and cooling. And then consisting of various heating and cooling stages of MD-based makes energy-minimize. C-docker uses soft-core potential to explore a variety of conformational fields of small organics and macromolecules. (Brooks, Bruccoleri, Olafson, States, Swaminathan, & Karplus,, 1983)

Cdocker energy is more positive score for choosing docked poses of each input ligands.

A higher Cdocker energy value indicates more favorable binding with small molecule and target protein. Receptor-ligand energy and internal ligand energy are in the Cdocker energy. This method gives more accurate results than others. (Brooks, Bruccoleri, Olafson, States, Swaminathan, & Karplus., 1983)

1.12 DE-NOVO

Denovo is most important ligand binding occurrences. The initial aim of new drug discovery is attempt to design new novel ligand compounds that are important target proteins therapeutically. New ligands are modified by changing the known inhibitors to increase the binding affinity of target proteins and ligands. Computational approaches to settling the inhibitor binding mode in target protein active site so called binding site, have been improved ((Friesner, et al., 2004)

De-novo drug design needs a three-dimensional structure of the target receptor protein. These methods used to design new ligand structures by serially adding new molecules to growing ligand structure. So functionality and molecular weight are changed comparatively. These methods can be used for creating different molecules structures. New ligands which are generated from de-novo producer are designed by adding fragments so this is crucial in ligand binding. New ligands are designed for increase the binding affinity and improve specificity of the ligands. (Denovo Protocol)

1.12.1 Fragment Docking Using De-Novo Receptor

Structure-based design is a founded method for using the receptor structure to discover new ligand. These protocols use the LUDI algorithm and prioritize lead candidates via the exploration of library of ligand scaffolds. (De novo Protocol)

1.12.2 Fragment Docking Using De-Novo Fragment

This method improves the ligand to the target receptor by finding appropriate fragments so it creates new ligands from the beginning ligand scaffold by Ludi Algorithm. (De novo Protocol)

2 Molecular Docking Setups

2.1 Docking setup of Autodock

In my thesis, firstly our study was asked to test the correct function of the system in order to determine the potential eNOS, iNOS and nNOS inhibitors based on structure. Selective inhibitors that only connected nNOS design are very difficult. For this purpose, the result of our system is tried to be a suitable by way of the various articles in the literature crystal structures of tested inhibitors in the available experimental data. So we use different docking software tools to determine the effective potential inhibitor and enzyme based on their binding affinity. AutoDock which is open source program, Libdock and C-Docker protocols in Accelrys software are used.

Four input parameter files are required for autodock so autodock tools creating pdbqt files which stores the atomic coordinates, partial charges and solvation parameters for all the atoms in the target proteins.,gpf files that is grid parameter files to set up Autogrid calculations and dpf files that creates docking parameter files for AutoDock calculations. Hydrogen atoms and gasteiger charges are added in pdbqt files. Grid files (gpf) include points of grid size, grid center points so grid files are defined as these parameters. In my thesis we use grid files as much as a number of enzymes. Grid center points of each

target enzymes on the x y z coordinates. Docking settings are designed for best docking. 70 70 70 grid points are chosen for three-dimension. Docking space is kept default 0.375 Å⁰. Dpf files contain docking algorithm. Number of energy evaluation of dockings, number of generation and number of runs of docking are defined in dpf files. These parameters of each docking that 1 chosen are in the Table 1.

Parameters of dpf file of Autodock	
Population size	150
Number of evaluation	5 000000
Number of generation	27000
Number of run	50-100

Table 1 : Parameters of Dpf files

2.2 Docking setup of Libdock

Libdock docking tools requires two input files. These files are target enzyme file and and ligand file that must be dsv format so at the beginning the pdb format for each enzymes are converted to the dsv format by using Accelrys. All NOS enzymes are accrossed this process. The binding site of target enzymes can use as volume of distance for all NOS enzymes. Therefore before the docking process binding site of each enzymes are defined. Radiuses of binding site of each enzyme are chosen 16 by using the position of known ligands so binding site volume is defined as spheres. Doing current selection is more important. Before the docking process, parameters are defined.

Libdock parameter is showed in Table 2. After the libdocking, libdock scores are ranked.

Parameters of Libdock	
Number of hot spots	100
Docking tolerance	0,25
Docking preferences	high quality
Conformation method	fast
Minimized algorithm	do not minimize

Table 2 : Parameters of Libdock

2.3 Docking setup of C-Docker

In CDOCKER, 10 replicas for each ligand are generated in the active site of the target receptor protein. The receptor binding site where ligand interact the receptor is created spherical shape with a diameter of 12Å⁰ and centered on the FAD molecule. Flexible ligand and rigid receptor are used for performing of simulated annealing. The ligand-protein interactions in the binding site are computed from grid extension 8.0. Random conformations are generated using 1000 molecular dynamic steps. In the simulated annealing, the number of heating step is set to 2000 steps, whereas cooling steps to 5000, and temperature of the heating is 700 K and temperature of the cooling is 300 K. The final refinement step of minimization is performed with full potential. Final minimized docking poses are clustered, based on a heavy atom. RMSD approach use 0.5 tolerance but the Discovery Studio 3.0 is analyzed the 10 top hit conformation.

Parameters of CDOCKER	
Top Hits	10
Random Conformations	10
Simulated Annealing	True

Table 3: Parameters of CDOCKER

2.4 Setup of De Novo

De novo receptor protocol was applied by using developed University of California ZINC library. 4 lead (scaffold) molecules that molecule for the enzyme produced from over 5000 molecules and that have high binding energy were selected. These scaffolds have the highest Ludi Score in the denovo protocols. Then these lead molecules are made fragment base de novo for modifying. Libdock and Autodock perform for each molecule.

1OM4, 3DQS and 1NSI enzymes are selected enzymes as a result of the Autodock and Libdock protocols. Leads that have high ludi score are selected for each enzyme. Potential inhibitory candidates are created using fragment-base de-novo. All ligands are docked to choose the best inhibitor using AutoDock and libdock. Results are discussed in result and discussion part.

When denovo protocols are used, we used an in stock subset of the following information. Zinc library compose of the third cluster in the database these are Standard, clean and in stock.

Standard subsets, numbers are 1-10. These are our approximations of popular subsets that appear commonly in the literature. Clean subsets numbers are 11-20. Immediate availability subsets, numbers are 21-30.

3 RESULT and DISCUSSION

3.1Preparation

In our study, we tested our system to determine the accuracy and precision of it by aiming to identify potential inhibitors based on the structures of nNOS (neural, in brain), eNOS (endothelial, inside veins) and iNOS (induced, in macrophage) enzymes but only nNOS selective ones.

Because all three enzymes have enormous similarities for the structure and shapes, it is very hard problem to design an inhibitor only binding to nNOS.

Therefore, the obtained results are fixed to the system by testing crystal structures of inhibitors whose experimental results are published on variety of related articles.

Initially, 3PNE, 3PNF, 3PNG, 3SVP, 3SVQ, 1OM4, 1P6I, 1P6J, 1QWC, 1RS7, 3B3N, 3B3O, 3DQR, 3N2R, 3SVP, 3B3M for nNOS, 3PNH, 1FOI, 3DQS, 3DQT for eNOS and 1NSI for iNOS are selected and retrieved from protein databank at the end of literature search.

Since our study will be carried on a subunit of 3D structure which is crystallized together with Arginine complex, proteins are converted to single domain chain.

Not only inhibitors but also water molecules and irrelevant molecules placed in the crystal complex obtained from protein databank are removed from the pdb files.

The charge of Fe atom which is placed in heme cofactor is changed to “+3” from “+2”.

The bond in between CYS amino acid which is generally located behind the Heme cofactor and Fe atom of Heme molecule is intentionally broken in Accelrys DS environment. Then, the hydrogen atoms are inserted. Distortions on the protein chain are corrected by the same program. Under the influence of a short minimization, the bond angles and distances are adjusted in an optimized manner. Hence, this optimized pro-structures became suitable to our docking (the insertion of ligands to the active sides of proteins) studies. (Figure 1 Original three-dimensional appearance of the eNOS enzyme(Figure 1Figure 2**Error! Reference source not found.**)

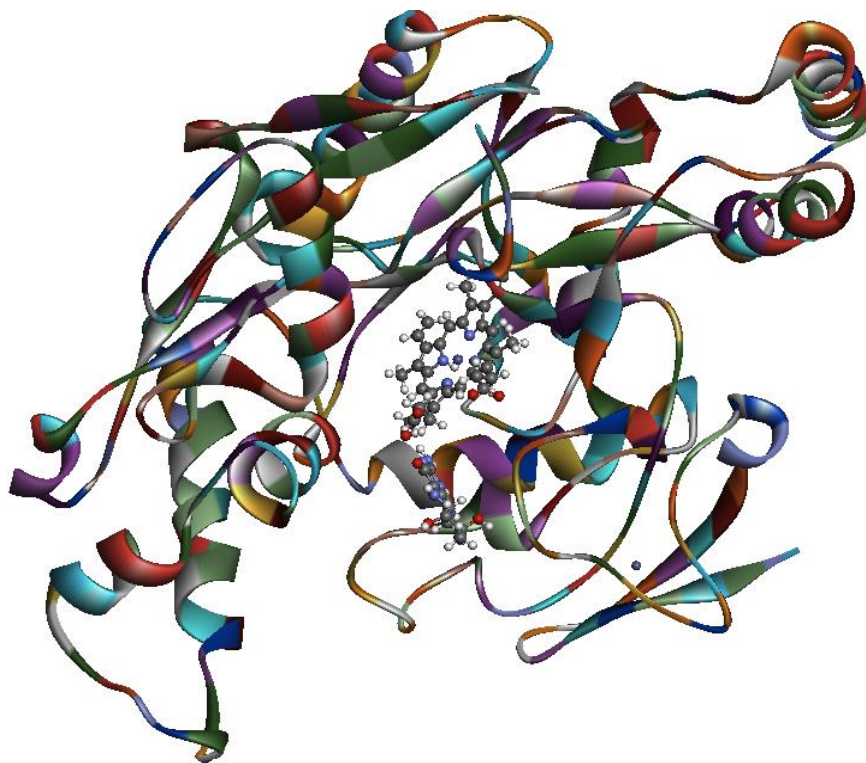


Figure 14: The original minimized 3D, single domain appearance of nNOS enzymes.

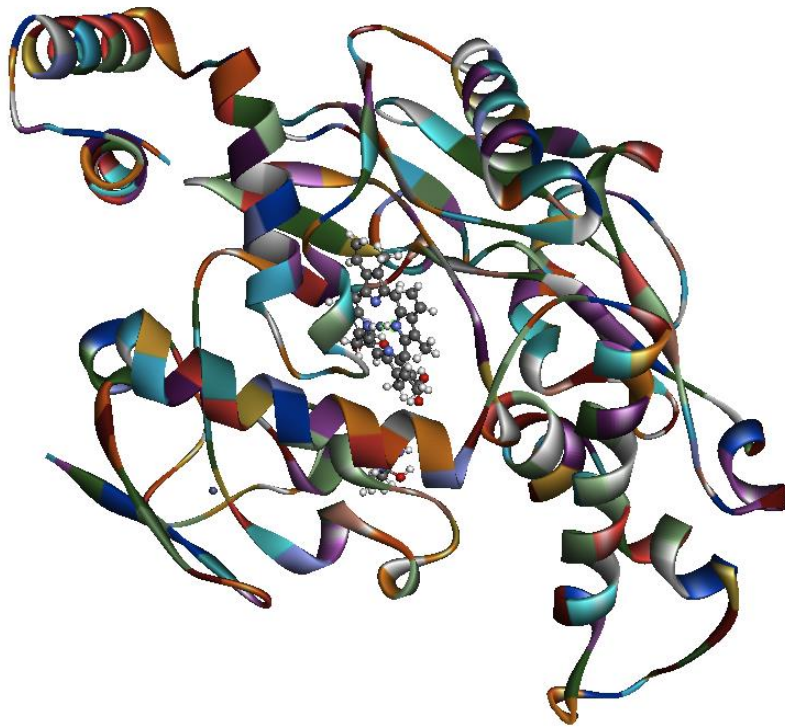


Figure 15: The original minimized 3D, single domain appearance of eNOS enzymes.

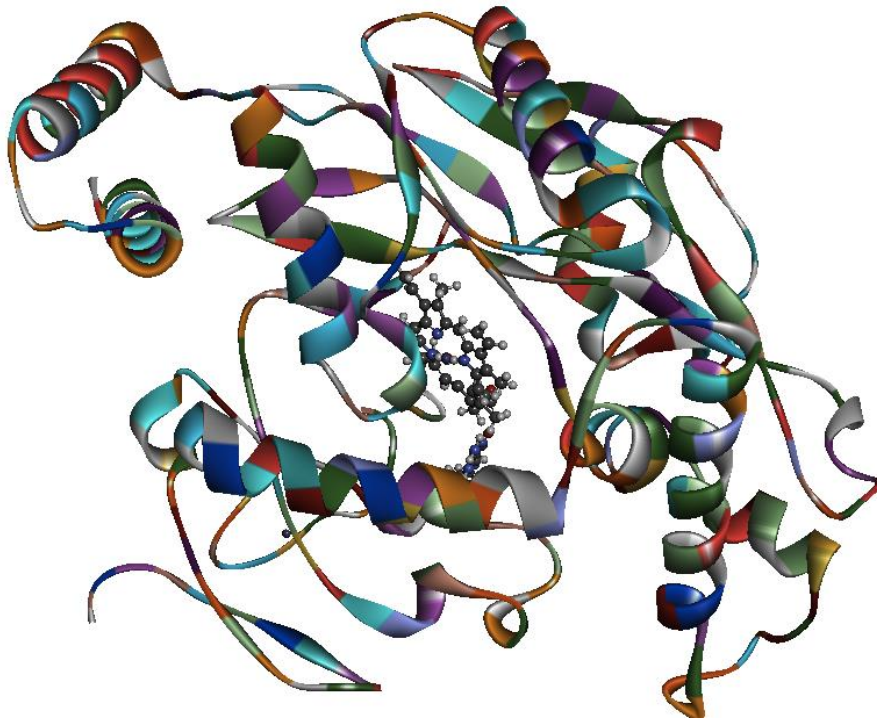


Figure 16: The original minimized 3D, single domain appearance of iNOS enzymes.

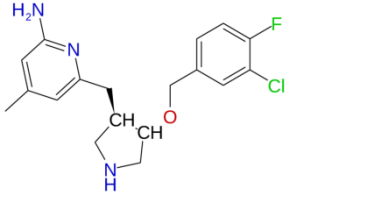
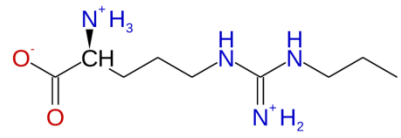
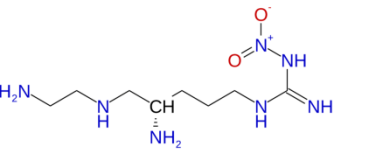
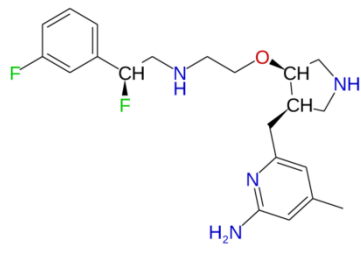
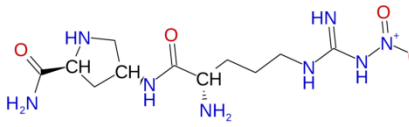
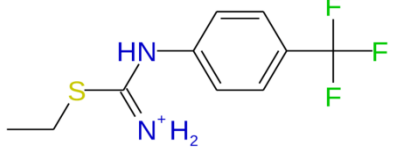
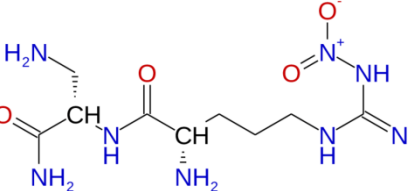
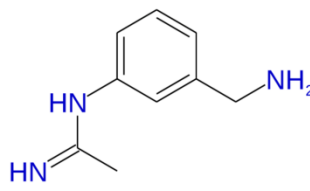
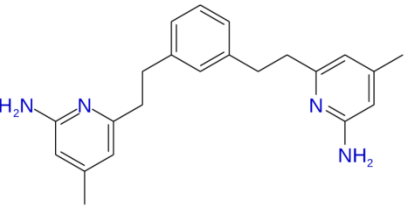
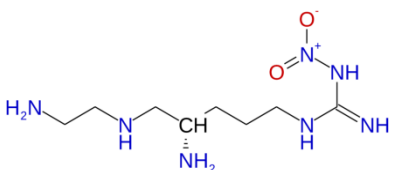
3.2 DOCKING

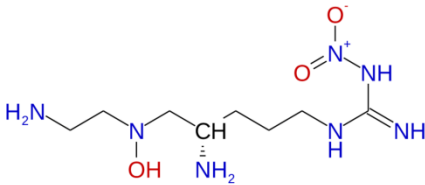
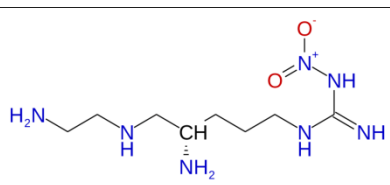
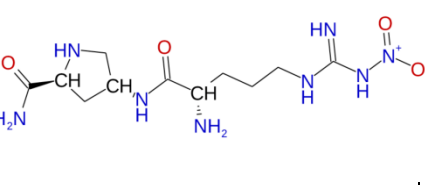
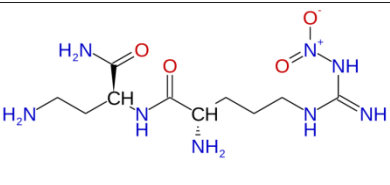
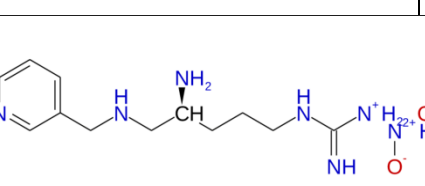
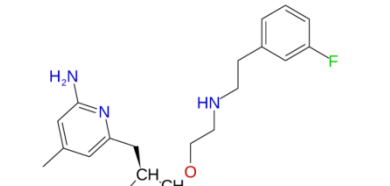
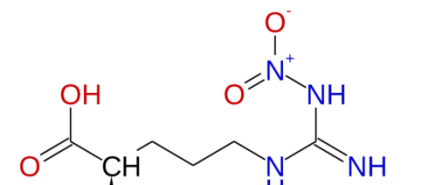
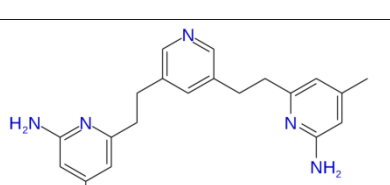
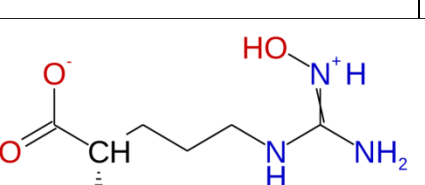
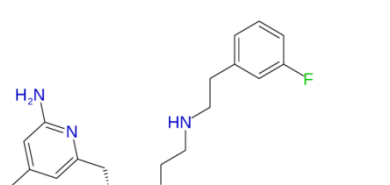
Compounds which are know their inhibition coefficient (Ki) are selected from different sources to enzymes. (Table 1). Structures have been minimized by drawing and computational binding energies were calculated using the Autodock 4.2 . Also Structure drawing program module and protocols in Accelrys Discovery Studio used for minimization.

Grid box dimensions 60 x 60 x 60 Angstrom, grid box center X, Y, Z coordinates and heme Fe atom that acted as a cofactor for each enzyme are chosen for inhibitors of the enzyme reach to the active site in Autodock study. For each of docking 10 independent runs is activated according to set 50 million for each of the evolution of energy.

Ki values that are obtained from Autodock4.2 and computational values are given in Table 2. Table 3 all values are converted logarithmic values. All results are nanomolar.

Table 4: Referenced 20 Compound that are compared with computational values

<p>1 Ref. 1</p>		<p>11 Ref. 10</p>	
<p>2 Ref. 2</p>		<p>12 Ref. 11</p>	
<p>3 Ref. 2</p>		<p>13 Ref. 12</p>	
<p>4 Ref. 3</p>		<p>14 Ref. 13</p>	
<p>5 Ref. 4</p>		<p>15 Ref. 14</p>	

<p>6 Ref. 5</p>	 <p>16 Ref.15</p>	
<p>7 Ref. 6</p>	 <p>17 Ref.16</p>	
<p>8 Ref. 7</p>	 <p>18 Ref.</p>	
<p>9 Ref. 8</p>	 <p>19 Ref.</p>	
<p>10 Ref. 9</p>	 <p>20 Ref.</p>	

3.3 Selection of Nnos Enzymes

Computational values of 20 compounds that are known their experimental values (Table 4) were found by docking to nNOS enzymes. Table 5 shows the 1OM4 nNOS enzyme that is the best fit with experimental data was chosen for modeling. Also graph plotted again as removed 3 computational our conclusions (10, 12 and 13 outliers) that are very difference according to experimental data. **(Figure 5)**

As can be seen in the figure a 1OM4 nNOS enzyme has become more compatible with assay.

Compound	3B3M	1OM4	1P6I	IP6J	1QWC	3B3N	3B3O	3DQR	3N2R	3SVP	Experimental
1	421,78	370,16	42,66	84,2	62,59	162,22	192,87	152,86	60,87	97,22	760
2	1710	59,57	1650	509,1	8,99	905,57	964,7	1030	265,79	8,53	120
3	12,97	14,58	6,36	44,96	0,1	5,97	16,86	145,3	10,61	1,06	100
4	368,53	217,86	702,81	959,27	2,29	439,24	1000	406	249,02	36,19	130
5	308,52	416,67	218,16	393,3	301,79	327,46	213,9	420,61	99,25	124,22	38
6	1960	64,54	1630	395,99	0,63	611,71	1370	210,79	603,38	14,39	120
7	194,02	0,05	13,9	33,44	0,11	222,36	53,19	136,84	37,99	8,79	100
8	157,5	47,49	99,08	276,64	0,7	269,51	573,45	855,42	134,67	22,74	50
9	1560	111,62	2680	3340	0,53	13310	3220	932,65	2810	37,02	600
10	652,11	30,54	361,54	990,85	1,49	1250	2630	2690	882,73	11,26	20000
11	431,56	17,22	248,48	947,27	1,33	1440	1810	2150	765,08	29,09	57
12	599,28	13570	561,42	1030	2940	366,09	263,8	4640	823,77	254,35	26
13	39240	60720	42370	70790	67590	49310	45800	27700	43720	31980	320
14	10590	6450	14780	10650	17030	8080	11510	22710	8310	6450	40
15	585,84	22,77	430,22	40,51	0,3	472,66	369,78	619,01	171,04	7,49	120
16	349,86	27,91	335,72	397,25	1,43	703,23	612,95	1010	171,97	4,94	170

17	6640	49,9	1340	941,75	4,22	1040	374,36	1530	652,15	2,05	130
18	3980	188,43	1070	630,45	1270	1880	1470	2410	477,17	566,21	15
19	1580	886,24	655,46	795,41	880,57	1260	338,52	324,3	807,45	249,89	25
20	960,71	863,54	171,28	286,39	2200	345,22	127,52	8660	874,6	1150	52

Table 5: Computational and Experimental inhibition constant of 20 compound and 10 nNOS enzymes from references. (All values are in nanomolar scale)

Compounds	3B3M	1OM4	1P6I	IP6J	1QWC	3B3N	3B3O	3DQR	3N2R	3SVP	Experimental
1	2,63	2,57	1,63	1,93	1,80	2,21	2,29	2,18	1,78	1,99	2,88
2	3,23	1,78	3,22	2,71	0,95	2,96	2,98	3,01	2,42	0,93	2,08
3	1,11	1,16	0,80	1,65	-1,00	0,78	1,23	2,16	1,03	0,03	2,00
4	2,57	2,34	2,85	2,98	0,36	2,64	3,00	2,61	2,40	1,56	2,11
5	2,49	2,62	2,34	2,59	2,48	2,52	2,33	2,62	2,00	2,09	1,58
6	3,29	1,81	3,21	2,60	-0,20	2,79	3,14	2,32	2,78	1,16	2,08
7	2,29	-1,30	1,14	1,52	-0,96	2,35	1,73	2,14	1,58	0,94	2,00
8	2,20	1,68	2,00	2,44	-0,15	2,43	2,76	2,93	2,13	1,36	1,70
9	3,19	2,05	3,43	3,52	-0,28	4,12	3,51	2,97	3,45	1,57	2,78
10	2,81	1,48	2,56	3,00	0,17	3,10	3,42	3,43	2,95	1,05	4,30
11	2,64	1,24	2,40	2,98	0,12	3,16	3,26	3,33	2,88	1,46	1,76
12	2,78	4,13	2,75	3,01	3,47	2,56	2,42	3,67	2,92	2,41	1,41
13	4,59	4,78	4,63	4,85	4,83	4,69	4,66	4,44	4,64	4,50	2,51
14	4,02	3,81	4,17	4,03	4,23	3,91	4,06	4,36	3,92	3,81	1,60
15	2,77	1,36	2,63	1,61	-0,52	2,67	2,57	2,79	2,23	0,87	2,08

16	2,54	1,45	2,53	2,60	0,16	2,85	2,79	3,00	2,24	0,69	2,23
17	3,82	1,70	3,13	2,97	0,63	3,02	2,57	3,18	2,81	0,31	2,11
18	3,60	2,28	3,03	2,80	3,10	3,27	3,17	3,38	2,68	2,75	1,18
19	3,20	2,95	2,82	2,90	2,94	3,10	2,53	2,51	2,91	2,40	1,40
20	2,98	2,94	2,23	2,46	3,34	2,54	2,11	3,94	2,94	3,06	1,72

Table 6: Logarithmic values of Computational and Experimental inhibition constant of 20 compound and 10 nNOS enzymes from references. (All values are in nanomolar scale)

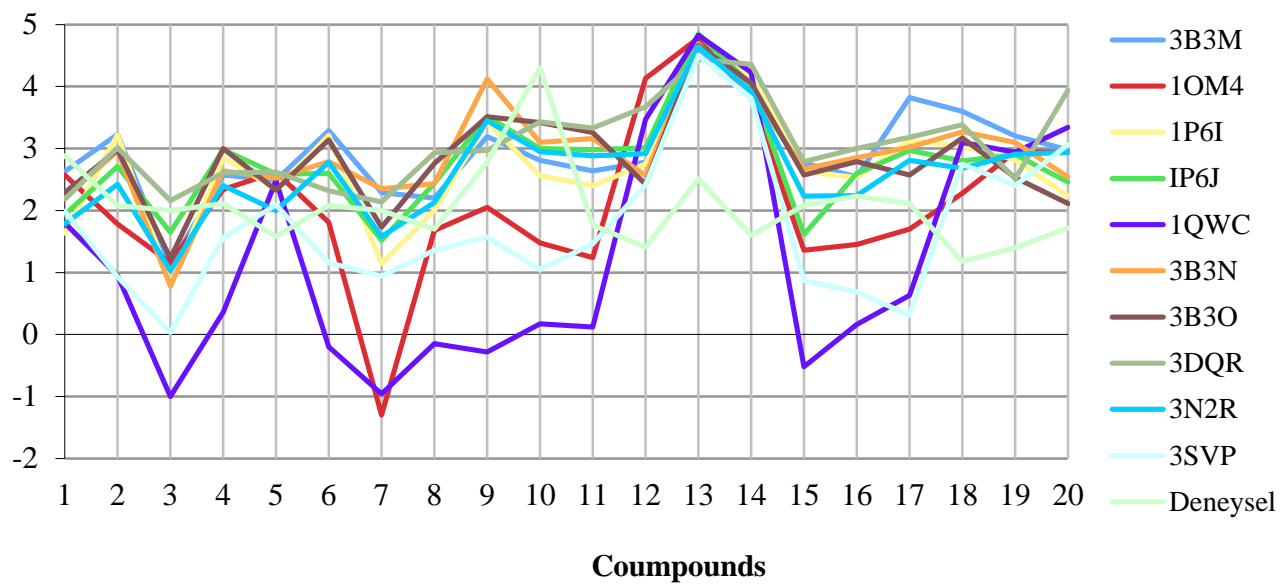


Figure 12 : Inhibition behavior of inhibition constant of 20 compound against the nNOS enzyme

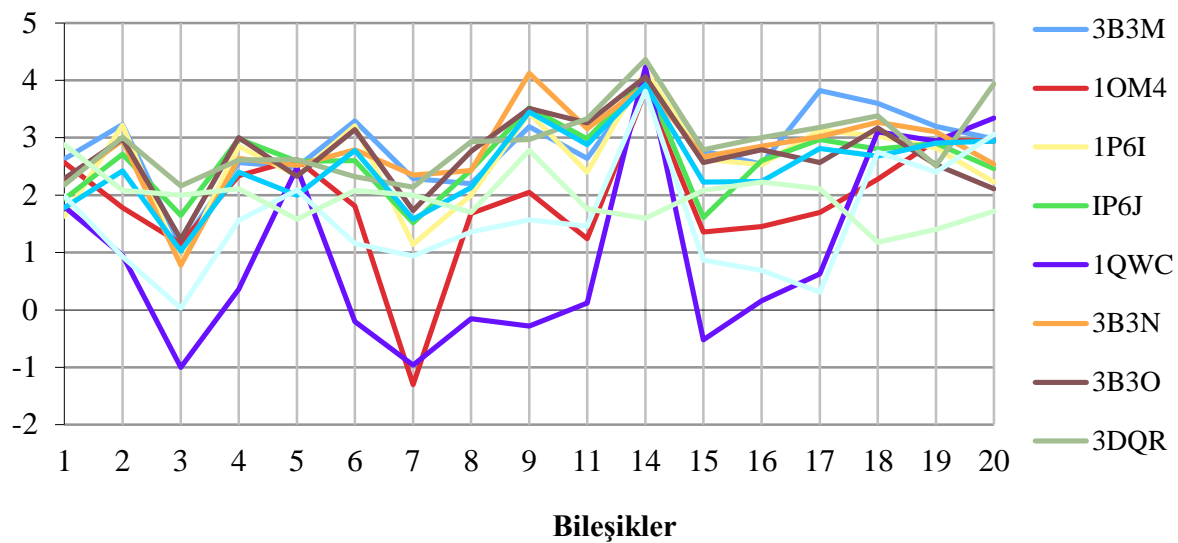


Figure 13: Inhibition behavior of inhibition constant of 20 compound against the nNOS enzymes. (10, 12, 13 compounds are extracted)

3.4 Selection of eNOS and iNOS

A 3DQS eNOS enzyme that is the best fit with experimental data was chosen according to the figure 6. INOS enzyme is the only one used in our studies so Inos minimized. Computational and experimental results show differences but it is very normal. In this study experimental compounds differ in the different studies. Enzymes results in different conformations can be changed again. In order to minimize error, making calculations with the average structure is the most accurate way.

Coumpounds	Experimentals	1FOI(eNOS)	3DQS(eNOS)	3DQT(eNOS)
1	13300	822,72	661,69	579,00
2	314000	119,45	736,55	1,32
3	128000	32,75	11,48	1,93
4	200000	718,31	701,88	2,49
5	4200	360,21	291,57	1030,00
6	73000	180,07	4600,00	1,94

7	128000	26,76	174,50	0,17
8	105000	34,44	170,85	0,73
9	1200	54,07	1930,00	0,10
10	8500	26,72	2240,00	0,28
11	19000	1470,00	551,51	1940,00
12	9400	45790,00	78250,00	61780,00
13	30	7980,00	5490,00	7640,00
14	314000	16,72	624,81	1,97
15	191000	82,56	3070,00	0,55
16	2000000	173,48	1730,00	1,21
17	31000	4340,00	848,41	727,16

Table 7: Experimental and Computational values of 17 compound and 3 eNOS enzymes that are selected references (All values are in nanomolar scale)

Coumpounds	Experimentals	1FOI(eNOS)	3DQS(eNOS)	3DQT(eNOS)
1	4,12	2,92	2,82	2,76
2	5,50	2,08	2,87	0,12
3	5,11	1,52	1,06	0,29
4	5,30	2,86	2,85	0,40
5	3,62	2,56	2,46	3,01
6	4,86	2,26	3,66	0,29
7	5,11	1,43	2,24	-0,77
8	5,02	1,54	2,23	-0,14
9	3,08	1,73	3,29	-1,02
10	3,93	1,43	3,35	-0,55
11	4,28	3,17	2,74	3,29
12	3,97	4,66	4,89	4,79

13	1,48	3,90	3,74	3,88
14	5,50	1,22	2,80	0,29
15	5,28	1,92	3,49	-0,26
16	6,30	2,24	3,24	0,08
17	4,49	3,64	2,93	2,86

Table 8: Logarithmic values of experimental and Computational values of 17 compound and 3 eNOS enzymes that are selected references (All values are in nanomolar scale)

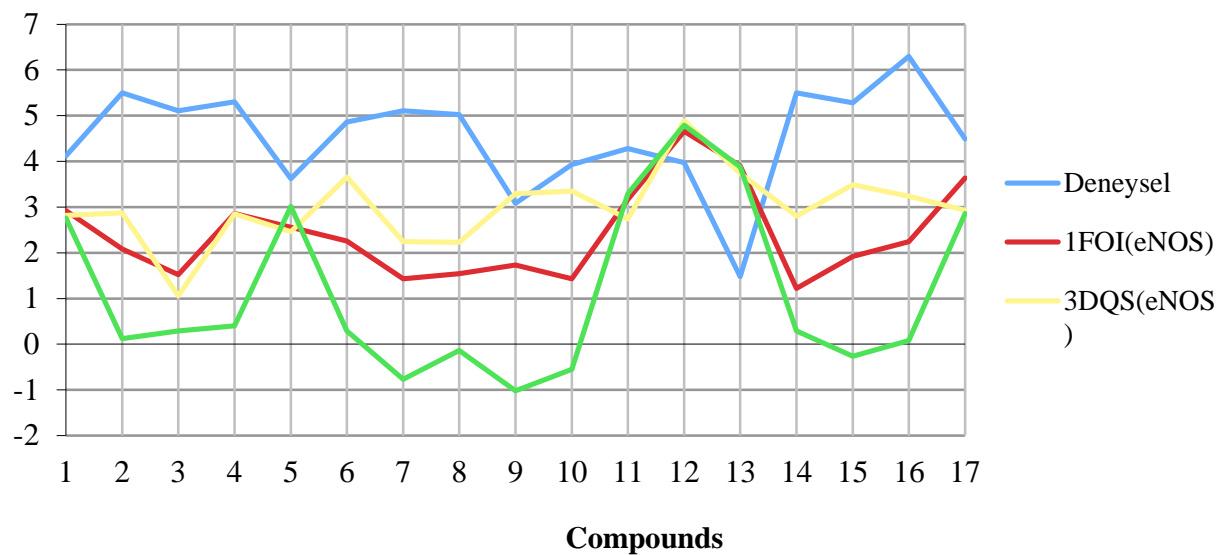


Figure 14 : Inhibition behavior of logarithmic constant of 17 compounds against the eNOS

Compound	Experimental	1NSI(iNOS)
1	50000	197,57
2	39000	0,04
3	29000	0,01
4	25000	0,09
5	2730	116,10

6	16000	0,30
7	29000	0,00
8	3510	0,11
9	14000	0,03
10	180000	0,07
11	26000	1900,00
12	37000	20430,00
13	670	55220,00
14	39000	0,14
15	18000	0,03
16	25000	0,07
17	9500	1890,00

Table 9: Experimental and Computational values of 17 compound and iNOS enzymes that are selected references (All values are in nanomolar scale)

Coumpounds	Experimental	1NSI(iNOS)
1	4,70	2,30
2	4,59	-1,37
3	4,46	-2,11
4	4,40	-1,05
5	3,44	2,06
6	4,20	-0,52
7	4,46	-2,44
8	3,55	-0,95
9	4,15	-1,56

10	5,26	-1,13
11	4,41	3,28
12	4,57	4,31
13	2,83	4,74
14	4,59	-0,84
15	4,26	-1,53
16	4,40	-1,14
17	3,98	3,28

Table 10: Logarithmic values of Experimental and Computational values of 17 compound and iNOS enzymes that are selected references (All values are in nanomolar scale)

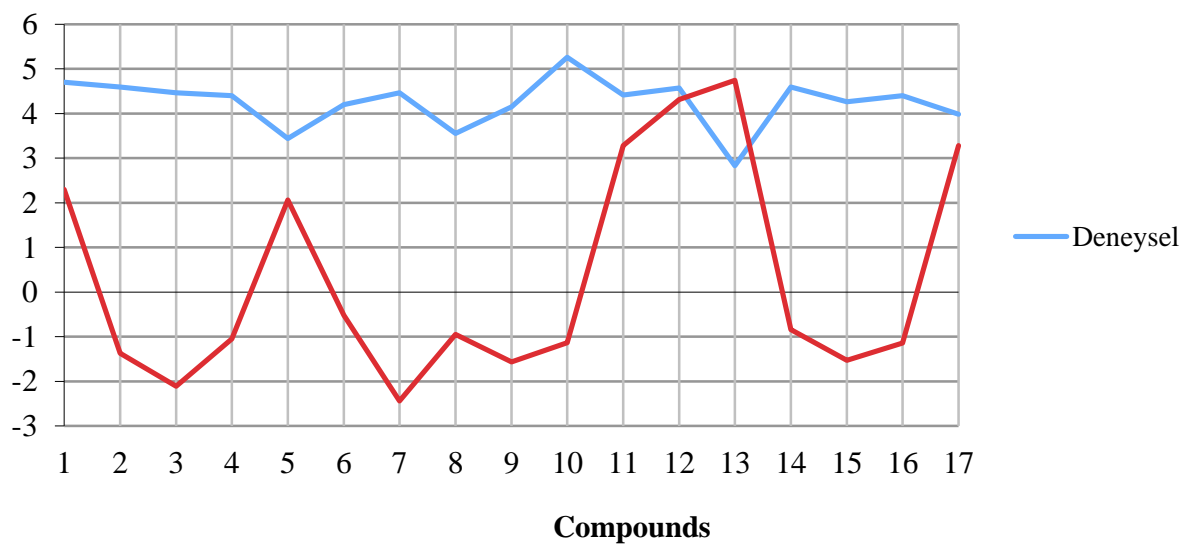


Figure 15 Inhibition behavior of logarithmic constant of 17 compounds against the iNOS

3.5 Comparison of Experimental and Computational Data

When the Table 5, Figure 13 are examined, experimental values of compound 9,12,13 and 14 higher than the experimental values of nNOS enzymes. Experimental values of compound 10 are significantly higher than the computational values. However the harmony was found for 1OM4 nNOS enzyme. As already mentioned graphic again draw by removing the three compounds (10, 12 and 13) that are very deviations at the figure 5. Experimental and computational data of 1OM4 shows that it is the most compliable enzymes. Consequently, 1OM4 neuronal enzymes will be used for computational studies.

3.6 nNOS Selective Inhibitor Design

As a result of our work, scaffolds were developed in the 1OM4 nNOS enzyme that is the most compliance with the experimental result.

De Novo receptor protocol was applied to 1OM4 nNOS enzyme by using Zinc library of California University.

4 lead scaffold molecules that have high binding energy with 1OM4 nNOS enzyme and have the highest score have been selected. **Computational** Later these scaffold are modified to produce 10 ligands by applying Denovo Fragment base library. Autodock program made for each of the docking in order to examine the layout of the three-dimensional, two-dimensional structure and select the scaffold in the produced ligands.

3.7 Scanning the inhibitor in the ZINC library

100 leads that are suitable with active site of nNOS enzymes produced using ZINCv12 library which includes one million molecules. Considering the De Novo Score and synthesizability 4 leads are selected for use fragment base de novo Table. From the chosen 4 potential lead, 200 potential inhibitor candidates were produced by using ZINCv12 library and library of Accelrys 3.1.fragment-based module. The candidates are selected from the 19 compounds considering the molecular weight, molecular structure and Denovo score Table 12

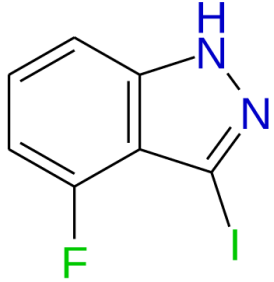
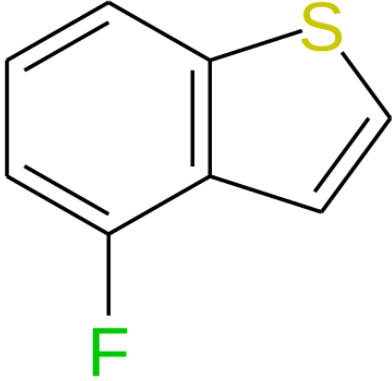
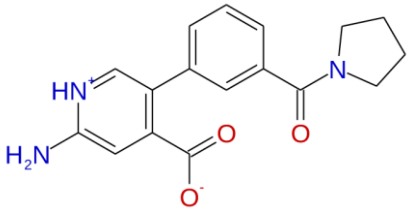
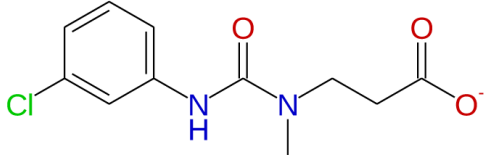
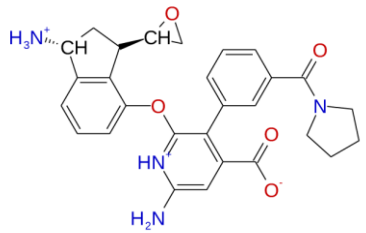
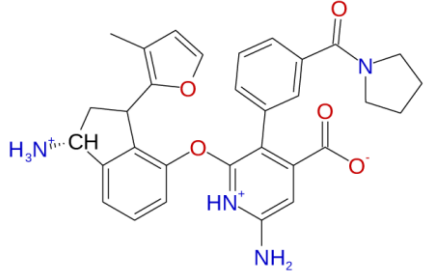
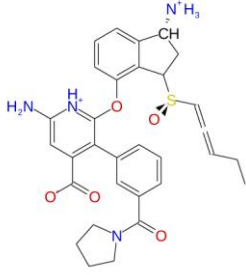
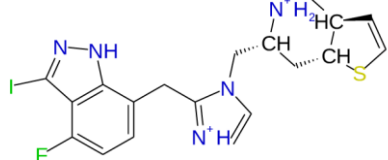
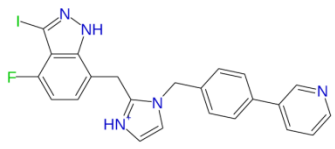
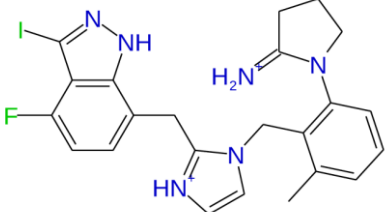
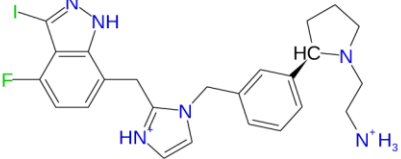
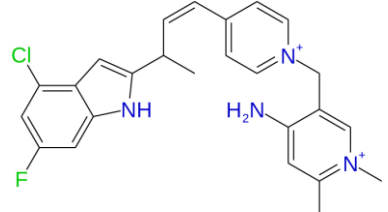
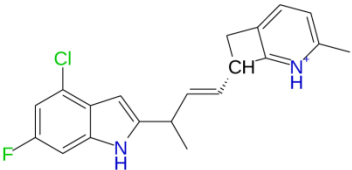
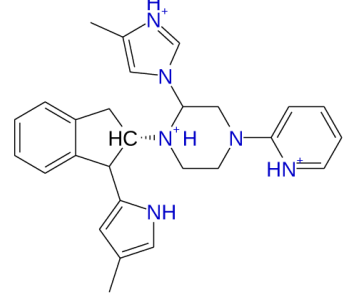
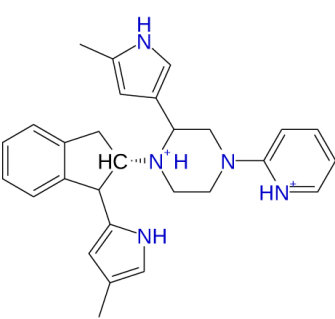
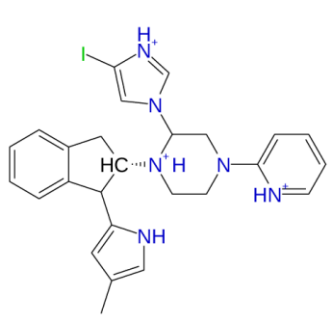
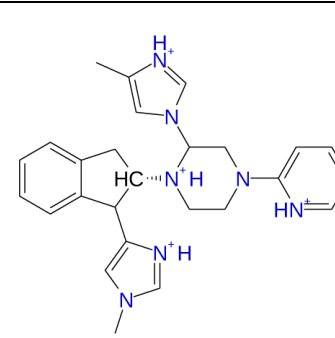
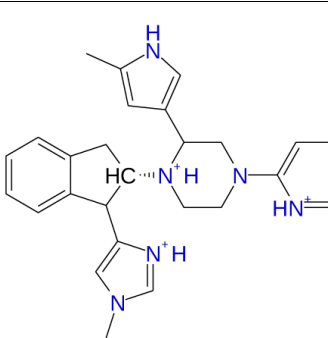
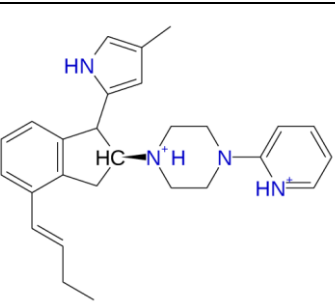
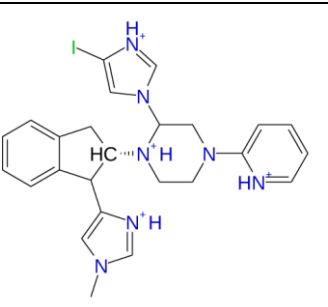
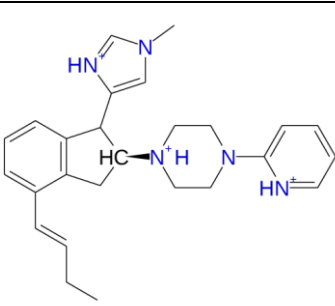
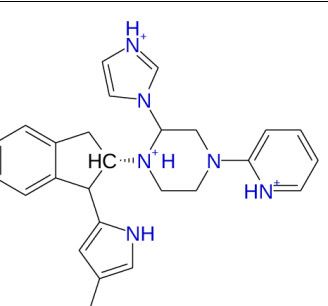
Lead1		Lead2	
Lead3		Lead4	

Table 11 The Leads used in this study

KN1		KN2	
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<p>KN3</p>		<p>KN4</p>	
<p>KN5</p>		<p>KN6</p>	
<p>KN7</p>		<p>KN8</p>	
<p>KN9</p>		<p>KN10</p>	

KN11		KN12	
KN13		KN14	
KN15		KN16	
KN 17		KN18	

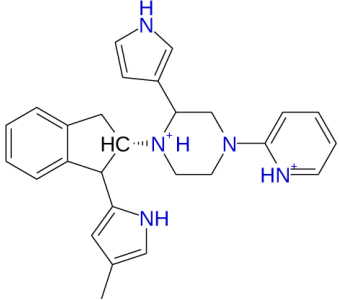
KN19	 <p>The chemical structure of KN19 features a central piperazine ring. One nitrogen atom of the piperazine is substituted with a 2-methylimidazole ring. The other nitrogen atom is substituted with a benzimidazole ring system, which consists of a benzene ring fused to an imidazole ring. A dashed line indicates a hydrogen bond between the hydrogen of the imidazole ring in the benzimidazole system and the nitrogen atom of the piperazine ring.</p>		
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Table 12 Selected inhibitor candidates



3.8 Docking Studies of Selected Inhibitors Candidates for nNOS, eNOS and iNOS Enzymes

Molecule No	nNOS Autodock	eNOS Autodock	iNOS Autodock	Molecule Weight
Inhibition Constant (nM)				
	nNos	eNOS	iNOS	
KN1	704.77	6310	5160	501
KN2	534.79	1370	3560	539
KN3	185.88	2920	1280	573
KN4	93.28	200.88	97.08	497
KN5	23.99	256.11	389.84	510

KN6	196.87	749.69	227.65	530
KN7	25.21	598.82	88.48	546
KN8	89.43	2420	505.29	436
KN9	734.32	3090	1550	341
KN10	20.22	131.72	129.91	441
KN11	19.97	145.77	129.56	439
KN12	20.18	145.01	133.51	553
KN13	20.11	134.45	129.73	443
KN14	20.23	145.07	130.61	441
KN15	19.9	135.43	128.52	414
KN16	20.35	146.33	132.5	555
KN17	20.4	129.75	137.59	416
KN18	20.28	146.85	131.64	427

KN19	20.11	137.29	133.21	425
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Table 13 Autodock results of obtained 19 inhibitors for eNOS, iNOS and nNOS enzymes

(All values are in nanomolar scales, Ki is inhibition constant)

	Inhibition Constant (nM)		
	nNos	eNOS	iNOS
KN1	2.848047409	3.800029359	3.712649702
KN2	2.728183278	3.136720567	3.551449998
KN3	2.269232664	3.465382851	3.10720997
KN4	1.969788537	2.3029367	1.987129768
KN5	1.380030248	2.408426536	2.590886398
KN6	2.294179541	2.874881718	2.357267655

KN7	1.401572846	2.777296297	1.946845114
KN8	1.951483231	3.383815366	2.703540703
KN9	2.865885357	3.489958479	3.190331698
KN10	1.305781151	2.119651722	2.113642583
KN11	1.300378065	2.163668154	2.112470939
KN12	1.304921162	2.161397953	2.125513796
KN13	1.303412071	2.128560807	2.113040418
KN14	1.305995883	2.161577611	2.115976429
KN15	1.298853076	2.131714878	2.108970717
KN16	1.308564414	2.165333373	2.122215878
KN17	1.309630167	2.113107367	2.138586871
KN18	1.307067951	2.166873951	2.119387874
KN19	1.303412071	2.137638905	2.124536828

Table 14 Logarithmic values of AutoDock results of obtained 19 inhibitors for eNOS, iNOS and nNOS enzymes (All values are in nanomolar scales, Ki is inhibition constant)

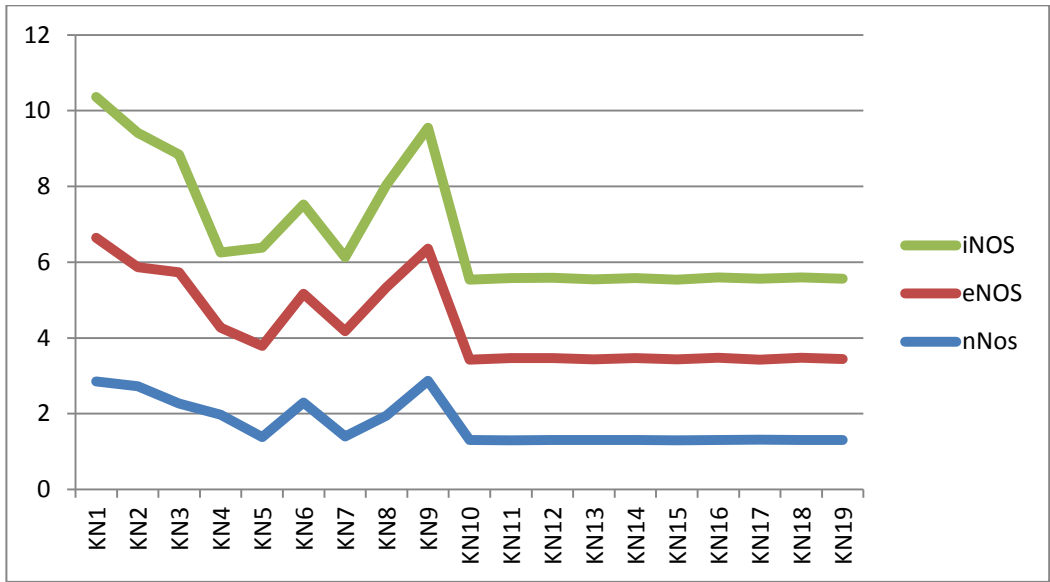


Figure 16 Graphical representation of logarithmic values in table 14

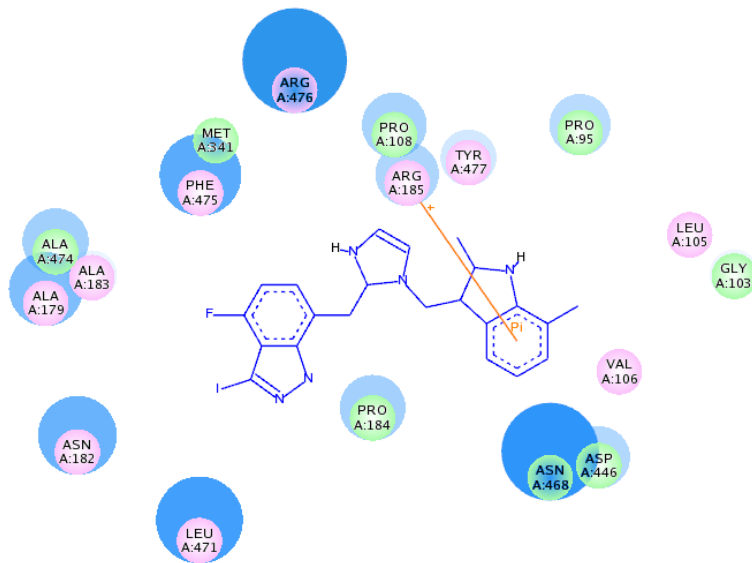
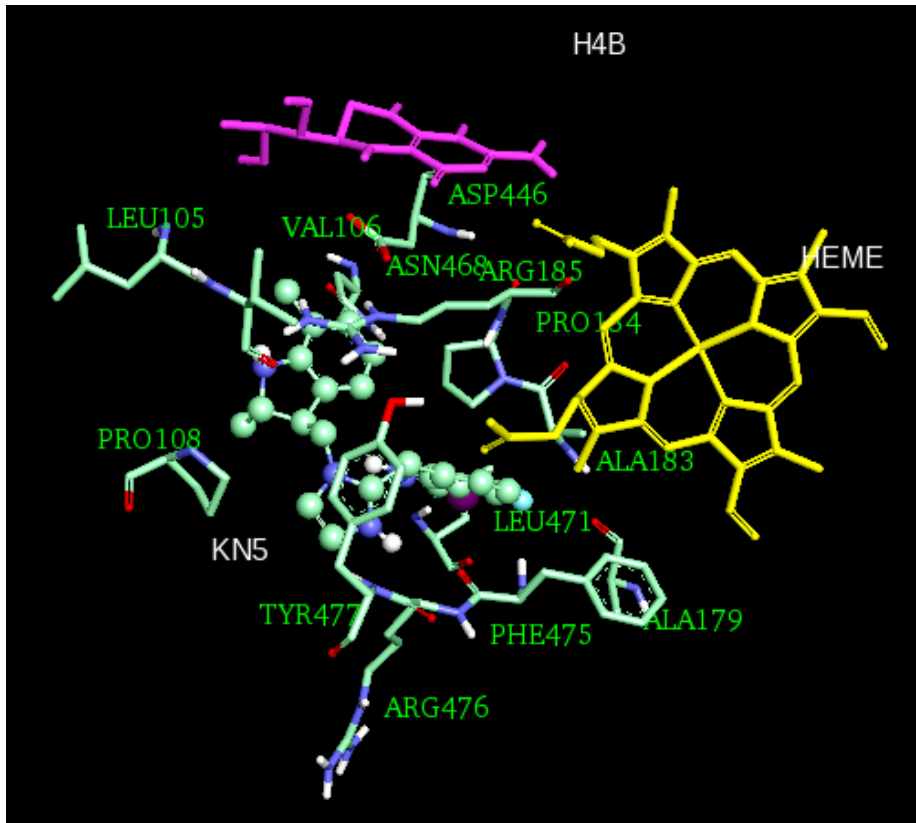


Figure 17 2D and 3D structures of KN5 Inhibitor in the eNOS enzyme

(Upper site) position of 3-D structure of KN5 inhibitor is shown in active site of eNOS enzymes. Amino acid side chains are shown in stick form and inhibitor in stick and circle form and also cofactor H4B (tetrahydrobiopterin) is shown in stick form and fuchsia color. Heme group is yellow.

(Lower site) Position of 2-D structure of eNOS site in the active site is shown. Hydrogen bonding or polar initiatives are shown fuchsia (magenta) colored circles and also hydrophobic and vdW interactions are shown as a green circle.

Blue circles that are around the crescent-shaped are located on the solvent accessible area.

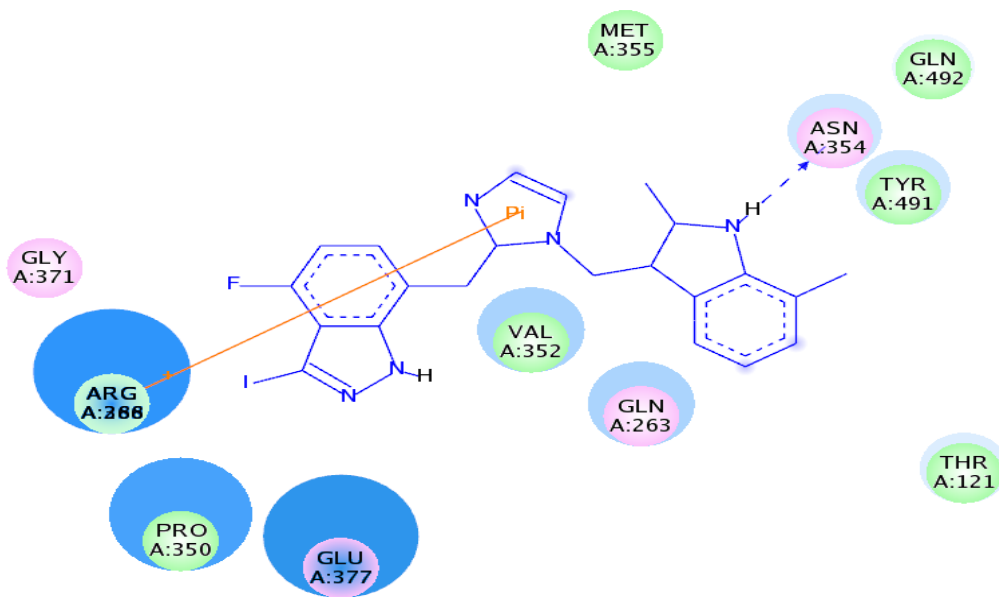
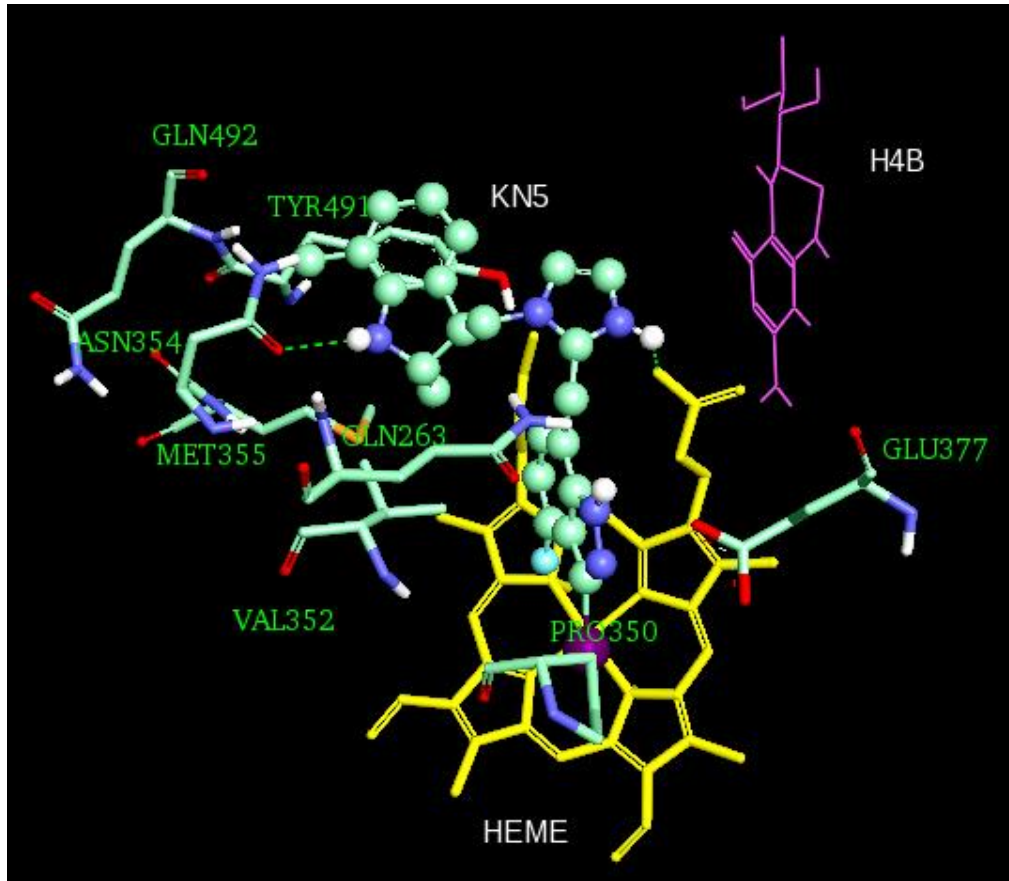


Figure 18 3D and 2D Structures of KN5 Inhibitor in the iNOS enzyme

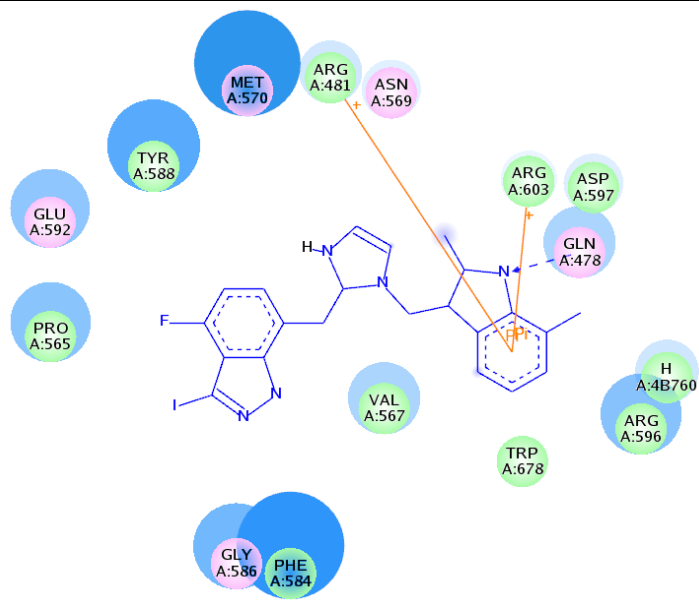
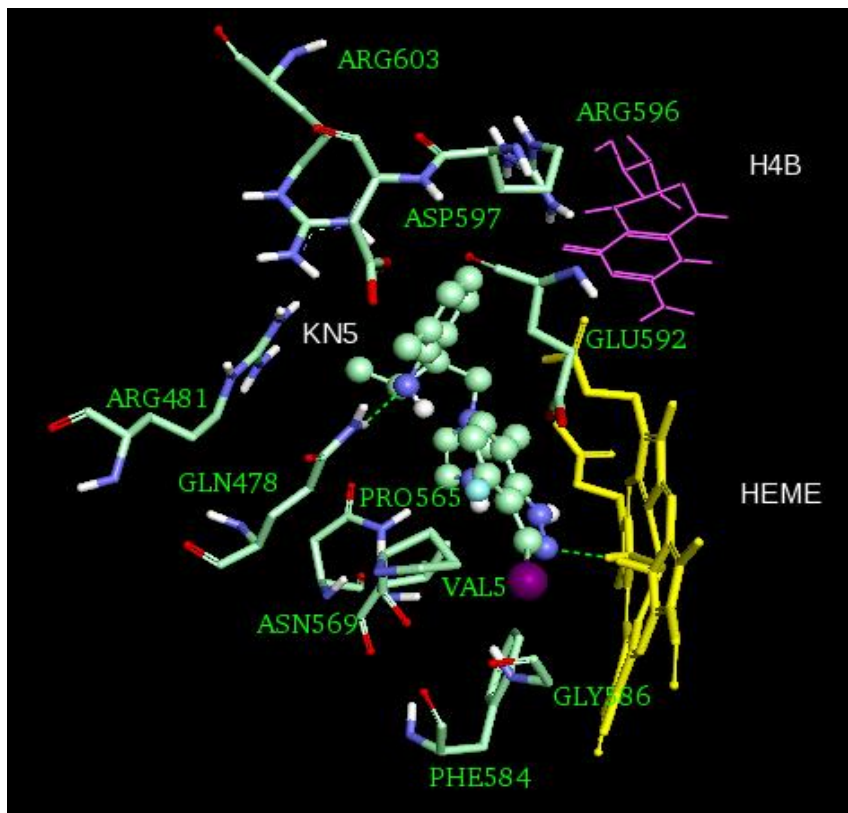


Figure 19 2D and 3D structure of KN5 Inhibitor in the nNOS enzyme

3.9 Result

For molecular modeling studies, we selected published ligands in the literature against the experimental values of the enzymes that are known. In our study, 1OM4 for nNOS, 1NSI for eNOS and 3DQS for iNOS isoenzymes that is compatible with the computational and experimental results have been identified. These enzymes have been used to improve new inhibitors for future studies.

Leads (scaffolds) that have highest affinity in the active sites of 1OM4 nNOS enzymes have been improved using De Novo receptor protocol of Accelrys. These four lead molecules were selected considering their molecular weights, de novo scores and synthesizability.

Utilizing Zinc and Accelrys de novo fragment libraries, binding affinities of lead were optimized in the 1OM4 active site by adding fragments. Obtained molecules were identified selectively nNOS by docking each three enzymes. Molecular structures and inhibition constant of the best 19 compounds are shown in Table 10 and Table 11.

As shown in figure 8, among the selected 19 inhibitors coded KN5 inhibitor show the best inhibition. Images of these compound binding poses in the active site of 3DQS, 1NSI and 1OM4 enzymes were given figure 9, figure 10 and figure 11 respectively as 2D and 3D.

As seen in figure 9 phenyl ring and ARG185 has attempted as a $\pi - \pi$. Inhibitor is surrounded by hydrophobic (VAL106, LEU105, ALA182, ALA179, PHE475) and polar (ASP446, ASN468, TYR477, ASN182) amino acids. On the other hand as seen in figure 10 the same inhibitor in the iNOS enzyme establish $\pi - \pi$ bond with histidine ring and ARG288 and it establish H-bond with N-H group of indole ring and ASN354. Ligand is surrounded by MET355, GLN492, TYR491, THR121, GLY371, PRO350 and GLU377. At figure 11 2D and 3D images of KN5 inhibitor are given. Here indane ring establish two $\pi - \pi$ bond with ARG653 and ARG481. In addition, N-H group of indane establish strong hydrogen bond with GLN478. There are ASN569, ASP597, ARG596, TRP678, PHE584, GLY586, PRO565, GLU592, TYR588 and MET570 amino acids around this bond. This binding mode explains why KN5 inhibitor which makes hydrogen and 2 $\pi - \pi$ bonds binds to nNOS enzymes with higher affinity.

nNOS enzyme not yet been tried, but with the method of docking and De Novo Design in Discovery Studio CH5, CH7, KN10 and KN11 have been developed as potential drug candidates. With docking and Renovo design in discovery studio technical drug candidates are developed such as KN5, KN7, KN10 and KN11 even though they have not been tested on nNOS yet. We think that this enzymes work on the nos enzymes by synthesis in the future project.

Bibliography

(Friesner, R. A., Banks, J. L., Murphy, R. B., Halgren, T. A., Klicic, J. J., Mainz, D. T., et al. (2004). Glide: A new approach for rapid, accurate docking and scoring. 1. *J. Med* , 1739-1749.

Abu-Soud, H. M., Presta, A., Mayer, B., & Stuehr, D. J. (1997). Analysis of Neuronal NO Synthase under Single-Turnover Conditions: Conversion of N^ω-Hydroxyarginine to Nitric Oxide and Citrulline. *Biochemistry* , 36, 10811-10816.

After, P. L., & Feldman. (1993). The surprising life of nitric oxide. *C&EN News* , 71, 26-38.

Arnet, U. A., McMillan, A., Dinerman, J. L., Ballermann, B., & Lowenstein, C. J. (1996). Regulation of Endothelial Nitric-oxide Synthase during Hypoxia. *The Journal Of Biological Chemistry* , 271 (25), 15069-15073.

AutoDock Protocol.

Autodock Tutorial.

Böhm, H. (1992). The computer program LUDI: A new method for the de novo design of enzyme inhibitors. *Comput - Aided Mol. Design* (6), 61-78.

Brooks, B. R., Brucoleri, R. E., Olafson, B. D., States, D. J., Swaminathan, S., &

Karplus, M. (1983). CDocker. *J. Comput. Chem.* (4), 187-217.

Denovo Protocol.

DISCOVERY STUDIO 3.0, protocols.

Erdal, E. P., Litzhger, E. A., Seo, J., Zuhu, Y., Ji, H., & Silverman, R. B. (2005).

Selective neuronal nitric oxide synthase inhibitors. *Curr Top Med Chem.* (5), 603-624.

Fengtian, X., James, K. M., Kristin, L. J., Ji, H., Mataka, J., Xia, G., et al. (2011).

Improved Synthesis of Chiral Pyrrolidine Inhibitors and Their Binding. *American Chemical Society*, 54, 6399.

Gachhui, R., Ghosh, D. K., Wu, C., Parkinson, J., Crane, B. R., & Stuehr, D. J. (1997).

Mutagenesis of Acidic Residues in the Oxygenase Domain of Inducible Nitric-Oxide Synthase Identifies a Glutamate Involved in Arginine Binding. *Biochemistry*, 36 (17), 5098.

GRID. (2002). *Version 20*. London, 4Chandos street, , U.K.: Molecular Discovery, Ltd.

Hah, J.-M., Martasek, P., Roman, L. J., & Silverman, R. B. (2003). Aromatic Reduced

Amide Bond Peptidomimetics as Selective Inhibitors of Neuronal Nitric Oxide Synthase. *J. Med. Chem.*, 46, 1661-1669.

Huang, P. L., Huang, Z., Mashimo, H., Bloch, K. D., Moskowitz, M. A., Bevan, J. A., et al. (1995). Hypertension in mice lacking the gene for endothelial nitric oxide synthase.

Nature , 377, 239-242.

Igarashi, J., Li, H., Jamal, J., Ji, H., Fang, J., Lawton, G. R., et al. (2009). Crystal Structures of Constitutive Nitric Oxide Synthases in Complex with De Novo Designed inhibitors. *J. Med. Chem.* , 52, 2060-2066.

Ji, H., Li, H., Flinspach, M., Poulos, T. L., & Silverman, R. B. (2003). Computer Modeling of Selective Regions in the Active Site of Nitric Oxide Synthases: Implication for the Design of Isoform-Selective Inhibitors. *J. Med. Chem.* , 46, 5700-5711.

Kowaluk, E. A., Daanen, J. F., Kohlhaas, K. L., Alexander, K. M., Wagenaar, F. L., Kerwin, J. F., et al. (1998). Nitroaromatic Amino Acids as Inhibitors of Neuronal Nitric Oxide Synthase. *J. Med. Chem.* , 41 (14), 2636--2642.

Lee, S., Oplinger, J. A., Frick, L. W., Garve, E. P., Furfine, E. S., & Shearer, B. G. (1997). Substituted N-Phenylisothioureas: Potent Inhibitors of Human Nitric Oxide Synthase with Neuronal Isoform Selectivity. *J. Med. Chem.* , 40, 1901-1905.

Li, H., Raman, C. S., Martasek, P., Masters, B. S., & Poulos, T. L. (2001). Crystallographic studies on endothelial Nitric Oxide complexed with nitric oxide and mechanism-based inhibitors. *Biochemistry* , 40 (18), 5399-5406.

Li, H., Shirnizu, H., Flinspach, M., Jamal, J., Yang, W., Xian, M., et al. (2002). The novel binding mode of N-Alkyl-N-Hydroxyguanidine to neuronal nitric oxide synthase provides mechanistic insights into NO biosyntheses. *Biochemistry* , 41, 13868-13875.

LibDock Protocol.

LIPINSKI@PFIZER.COM. (n.d.).

Olken, N. M., Osawa, Y., & Marletta, M. A. (1994). Characterization of the Inactivation of Nitric Oxide Synthase by NG-Methyl-L-arginine: Evidence for Heme Loss.

Biochemistry , 33 (49), 14784-14791.

Poulos, T. L., Silverman, R. B., Jamal, J., Li, H., Xue, F., & Delker, S. L. (2010). Role of Zinc in Isoform-Selective Inhibitor Binding to. *Biochemistry* , 49 (51), 10803--10810.

RA, C., Congreve, M., Murray, C., & Rees, D. (2005). Fragment-based lead discovery: leads by design. *Drug Discov Today* (10), 987-992.

RA, C., Congreve, M., Murray, C., & Rees, D. (2005 Jul 15). Fragment-based lead discovery: leads by design. *Drug Discov Today* , 987-992.

Schulz, R., Nava, E., & Moncada, S. (1992). Induction and potential biological relevance of a Ca²⁺ -independent nitric oxide synthase in the myocardium. *Br. J. Pharmacol.* , 105, 575-580.

Shearer, B. G., Oplinger, J. A., Lee, S., Garvey, E. P., Salter, M., Duffy, C., et al. (1998). N-Phenylamidines as Selective Inhibitors of Human Neuronal Nitric Oxide Synthase: Structure-Activity Studies and Demonstration of in Vivo Activity. *J. Med. Chem.* , 41 (15), 2858-2871.

Silverman, R. B. (2009). Design of selective neuronal nitric oxide synthase inhibitors for the prevention and treatment of neurodegenerative diseases. *Accounts of Chemical Research* , 42 (3), 439-451.

Silverman, R. B., Martasek, Roman, Huang, & Hui. (2000). Synthesis and Evaluation of Peptidomimetics as Selective Inhibitors and Active Site Probes of Nitric Oxide Synthases. *J. Med. Chem.* , 43, 2938-2945.

Silverman, R. B., Poulos, T. L., Roman, L. J., Martásek, J., Xue, F., Huang, J., et al. (2010). Structure-based design, synthesis, and biological evaluation of lipophilic-tailed. *Bioorganic & Medicinal Chemistry* , 18, 6526--6537.

Silverman, R. B., Roman, L. J., Martasek, P., Gomez-Vidal, J. A., & Ji, H. (2006). Conformationally Restricted Dipeptide Amides as Potent and Selective Neuronal Nitric Oxide Synthase Inhibitors. *J. Med. Chem.* , 49 (21), 6254-6263.

Silverman, R. R., Seo, J., Igarashi, J., Li, H., Martasek, P., Poulos, T. L., et al. (2007). Structure-Based Design and Synthesis of N^o-Nitro-L-Arginine-Containing Peptidomimetics as. *J. Med. Chem* , 50 (9), 2089-2099.

Silverman,, R. B., Ji, H., Stanton,, B. Z., Igarashi,, J., Li., H., Martasek,, P., et al. (2008). Minimal Pharmacophoric Elements and Fragment hopping, an approach directed at Molecular Diversity and Isozyme Selectivity. Design of Selective Neuronal Nitric Oxide Synthase Inhiitors. *J.Am.Chem. Soc.* , 130, 3900-3914.

- Tierney, D. L., Huang, H., Martasek, P., Roman, L. J., Silverman, R. B., & Hoffman, B. M. (2000). ENDOR spectroscopic evidence for the geometry of binding of retro-inverso-N ω -nitroarginine-containing dipeptid amides to neuronal nitric oxide synthase. *J. Am. Chem. Soc.* , 122, 7869-7875.
- Westerhuis, J. A., Kourti, T., & Macgregor, J. F. (1998). Analysis of multiblock and hierarchical PCA and PLS models. *J. Chemom.* (12), 301-321.
- Wolff, D. J., Gauld, D. S., Neulander, M. J., & Southan, G. (1997). Inactivation of Nitric Oxide Synthase by Substituted Aminoguanidines and Aminoisothioureas. *The Journal Of Pharmacology and Experimental Therapeutics* , 283 (1), 265-273.
- Zhang, H. Q., Fast, W., Marletta, M. A., Martasek, P., & Silverman, R. B. (1997). Potent and Selective Inhibition of Neuronal Nitric Oxide Synthase by N ω -Propyl-L-arginine. *J. Med. Chem.* , 40, 3869-3870.