KADIR HAS UNIVERSITY

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IN SILICO SCREENING OF NEURONAL NITRIC OXIDE SYNTHASE ENZYME INHIBITORS

GRADUATE THESIS

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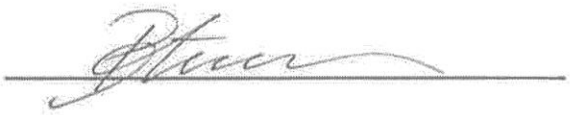

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ABSTRACT

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Master of Science in Computational Biology and Bioinformatics

May, 2014

Three closely related isoforms of nitric oxide synthases (NOS) catalyze an important secondary messenger nitric oxide (NO) synthesis through oxidation of L-arginine to L-citrulline. These three NOS isoforms takes parts in different tissues for various physiological and pathological processes. Neuronal NOS (nNOS) produce NO in central and peripheral nervous system, endothelial NOS (eNOS) plays role in endothelial cells and NO in macrophage cells is produced by inducible NOS (iNOS). Excessive NO production in nervous cells following pathological conditions is observed. Dysregulation of NO, therefore, may force NO to act as a neurotoxin that causes several neurodegenerative diseases including Parkinson's, Alzheimer's, Huntington's diseases. Considering all these facts, developing a selective and good potential inhibitor for nNOS is a compulsory task to achieve. However, among all

isoforms there is high active site conservation so that no drug that shows these desired properties has yet been designed and developed.

In this present work, virtual screening techniques were applied to design selective nNOS inhibitors. Molecular modeling studies were done using already known crystal structures of all three isoforms. First of all, to find primary lead candidates, several hundred compounds were screened via ZINCv12 lead library. Then, modifications were done on the selected scaffolds via *de novo* design method to derive our inhibitor candidates. AutoDock 4.02 docking virtual tool was employed for docking and scoring of inhibitor candidates. Inhibition constants and best pose predictions of docked ligands within the active sites of three isoforms were considered for further examinations and comparison analysis. Already bound ligands in downloaded experimentally determined X-ray structures of all isoforms were re-docked to crosscheck our studies. In this thesis two lead scaffolds among all and 22 inhibitor candidates derived from these two scaffolds were selected to discuss for optimization for further development of best potential and selective inhibitor for nNOS.

Keywords: Nitric Oxide Synthase, eNOS, iNOS, nNOS, *de novo* design, docking, *in silico* screening

SEÇİCİ NİTRİK OKSİT SENTAZ ENZİM İNHİBİTÖRLERİNİN BİLGİSAYAR ORTAMINDA TARANMASI

ÖZET

BAHANUR ÖRTMEN

Hesaplamalı Biyoloji ve Biyoinformatik, Yüksek Lisans

May, 2014

Nitrik oksit sentaz enziminin (NOS) üç izoformu, L-arjininin L-sitruline oksidasyonu ile önemli bir ikincil haberci olan nitric oksit (NO) sentezler. NOS'un bu üç izoformu çeşitli fizyolojik ve patolojik süreçte farklı dokularda yer almaktadır. Nöronal NOS (nNOS) merkezi ve periferik sinir sisteminde, endotelyal NOS (eNOS) endotel hücrelerinde ve indüklenebilen NOS (iNOS) ise makrofaj hücrelerinde NO sentezini katalizlemektedir. Parkinson, Alzheimer, Huntington hastalığı gibi çeşitli nörodejeneratif hastalıklarda aşırı NO üretimi görülebilir ve uygunsuz NO regülasyonu, NO'nun nörotoksin gibi davranmasına neden olabilir. Tüm bu bulgular değerlendirildiğinde, nNOS'a seçici ve nNOS üzerinde güçlü inhibitör etki gösteren ilaçların geliştirilmesinin gerekli olduğu görülmektedir. Ancak NOS izoformlarının aktif bölgesinde yüksek derecede benzerlik bulunmasından dolayı bu istenilen özellikleri gösteren bir ilaç henüz tasarlanmamış ve geliştirilmemiştir.

Bu yapılan çalışmada, selektif nNOS inhibitörü tasarlamak amacıyla bilgisayar ortamında sanal tarama teknikleri uygulanmıştır ve NOS'un üç izoformunun bilinen kristal yapıları kullanılarak moleküler modelleme çalışmaları yapılmıştır. İlk olarak, öne çıkan küçük molekül iskeletlerini bulmak için ZINCv12 parçaçık kütüphanesi aracılığı ile yüzlerce bileşik taranmıştır. Daha sonra, inhibitör adaylarının türetilmesi için *de novo* tasarım yöntemleriyle seçilen modellerde modifikasyonlar yapılmıştır. Adayların inhibitör derecesi ve inhibisyon bölgesine hedeflendirilmesi için Autodock 4.02 hedeflendirme aracı kullanılmıştır. İnhibisyon katsayıları ve hedeflendirilen ligandın üç izoformun aktif bölgelerindeki en iyi konumu tetkik ve karşılaştırma analizleriyle değerlendirilmiştir. Çalışmalarımızı doğrulamak için izoformların deneysel sonuçlardan elde edilen X-ışını yapılarına bağlı ligandlar AutoDock ile yeniden hedeflendirilmiştir. Bu çalışmada tasarlanan öncü iskeletlerden 2 tanesi ve bu 2 iskeletten türetilen inhibitor adaylarının 22 tanesi, en güçlü selektif nNOS inhibitörü optimizasyonunun tartışılması için belirlenmiştir.

Anahtar Kelimeler: Nitrik oksit sentaz, eNOS, iNOS, nNOS, *de novo* dizayn, ilaç hedeflendirmesi (docking) , in silico tarama

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1 INTRODUCTION

1.1 Nitric Oxide Synthase and Nitric Oxide

Over three decades, structure of nitric oxide synthase, function of nitric oxide synthases (NOS) and inhibition of NOS enzyme are important subjects for many researches since it synthesizes an important signaling molecule in various tissues, nitric oxide (NO). [1] NOS catalyzes a NADPH- dependent formation of NO and citrulline from L-arginine. [2, 3] (**Figure 1**)

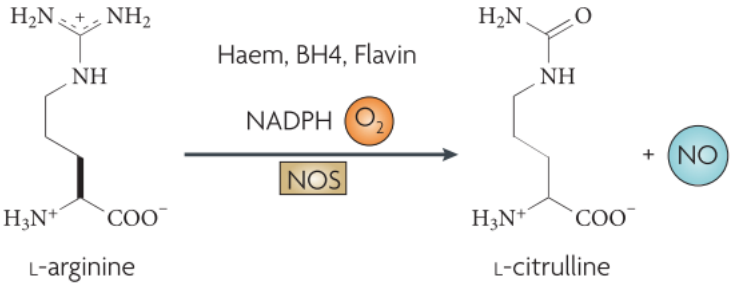


Figure 1 | **Chemical Reaction of NO synthesis carried by NOS.** [3]

This free radical gas, nitric oxide is produced essentially in endothelials, macrophages and neuronal cells with the reaction in **Figure 1** carried by three isoforms of NOS enzyme; endothelial NOS (eNOS), neuronal NOS (nNOS) and macrophagal or inducible NOS (iNOS). nNOS and eNOS are constitutively active

and Ca^{2+} - dependent isoforms. Whereas there is another isoform Ca^{2+} independent that depends on mechanism of action, inducible NOS (iNOS). [4]

All three isoforms take part in many important physiological and pathophysiological processes in mammalian cells. iNOS- derived NO in macrophage cells has important role as cytotoxic agent to destroy pathogens and microorganisms during immune and inflammatory response. eNOS-derived vascular NO plays significant role in controlling vascular protection such as blood pressure, protection from platelet aggregation. [5] Main function of nNOS-derived NO is releasing neurotransmitters and nNOS-derived NO has been indicated in **Figure 2** showing central effects and peripheral effects. The nNOS-derived NO has important roles in various synaptic signaling, synaptogenesis events and in modulation of actions such as learning, memory and neurogenesis. [6, 7, 8]

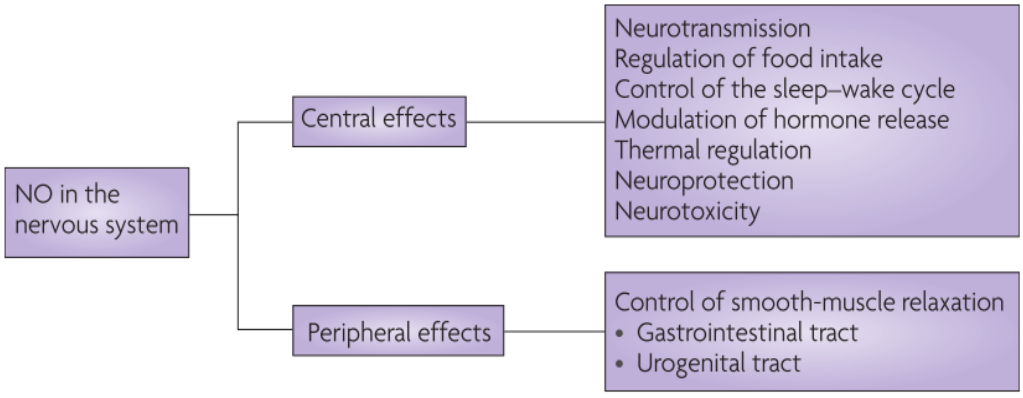


Figure 2 | **Roles of Nitric Oxide in Central and Peripheral Nervous System.** [8]

1.2 Structure of Nitric Oxide Synthases

Common three isoforms of NOS are inducible NOS, endothelial NOS and neuronal NOS. In different chromosomes, there are three distinct genes coding for three isoforms. *NOS1* gene corresponding to nNOS protein has 29 exons and 28 introns found on chromosome 12. *NOS2* gene corresponding to iNOS found on chromosome 17 and *NOS3* gene corresponding to eNOS found on chromosome 7 with 26 exons and 25 introns. However, all isoforms have almost same genomic structures. [1] NOS enzymes are generally found as dimer structure and each monomer generally consist of 420 to 430 amino acids. In **Figure 3**, amino acid sequences of one domain of all isoforms were aligned to show similarity. Different brightness of green color shows similarity degrees. For this alignment and for our project, PDB structures with 1OM4 code for nNOS structure, 3DQS for eNOS structure and finally 1NSI for iNOS are used.

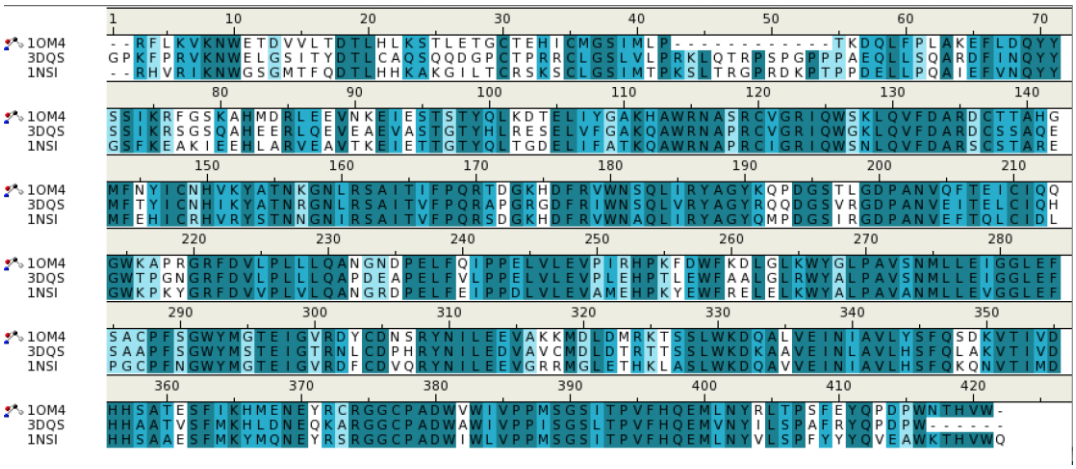


Figure 3 | **Alignment of Amino Acid Residues of Three NOS Isoforms.** Dark green colors correspond to exact matching residues; light green colors show partial similarity and white colors correspond to mismatch residues. (Isoforms were aligned using Discovery Studio 3.0)

In **Figure 4**, only one domains of isoforms were excreted and backbones of these domains superpositoned to show structural homology between isoforms. All isoforms consist oxygenase domain in amino-terminal of protein and reductase domain in carboxy-terminal and also between these domains there is a calmodulin-binding region. Flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and NADPH binding sites are found in C-terminal reductase domain. And in N-terminal oxygenase domain, there are L-arginine, heme and BH_4 binding sites. (**Figure 5**) [9,10,11]

Figure 4 | **Superposition of Chain A Backbone of 1OM4(nNOS), 3DQS(eNOS) and 1NSI(iNOS).** Yellow, green and magenta colors correspond to 1OM4, 3DQS and 1NSI respectively. (Isoforms were superpositioned using Discovery Studio 3.0)

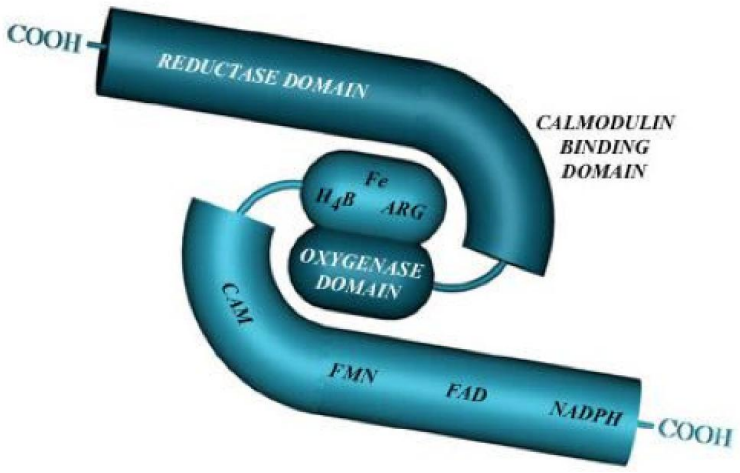


Figure 5 | **General Structure of NOS enzymes.** Structure is shown in as dimerized enzyme. Monomer at the top shows domains of NOS, monomer at the bottom highlights binding sites found on domains. [9]

1.3 Regulations and Function of Nitric Oxide Synthases

In all isozymes, flavins FMN, FAD and BH_4 play role as cofactors in the mechanism of nitric oxide synthesis. An Active NOS enzyme transfers electrons from NAPDH to flavins, FAD and FMN in carboxy-terminal reductase domain and then, as a result of conformational changes followed by calmodulin binding, electrons are transferred to heme in amino-terminal oxygenase domain. These transferred electrons are used to reduce molecular oxygen to superoxide (O_2^-) and L-arginine is oxidized to L-citrulline, then NO is produced in oxygenase domain. Heme is important for dimerization of NOS enzymes to set functional enzyme and thereby takes part in BH_4 and L-arginine binding. And calmodulin, which binds a region between reductase and oxygenase domain, promotes for electron transfer. (**Figure 6, A** and **B**) [12 13]. Constitutive and inducible NOS enzyme isoforms are differentiated at this point. Calmodulin binding is promoted by increased level of intracellular Ca^{2+}. Whereas, inducible NOS contains irreversibly controlled mechanism of CaM binding. So no intracellular Ca^{2+} is needed for CaM binding during NO synthesis by iNOS.[9,12,13]

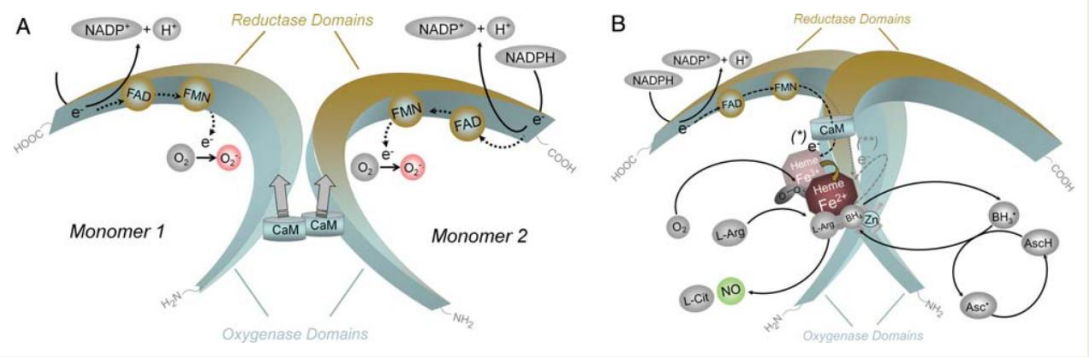


Figure 6 | Mechanism **of Nitric Oxide Synthesis (A)** Monomers involve in electron transfer from reduced NAPDH to both FAD and FMN. This e^- flow results in reducing molecular oxygen to O^-_2 (superoxide). CaM binding to reductase domains of monomers supports electron transfer within resuctase domain. **(B)** Heme forces monomers to form dimer structure and it is important for interdomain electron flow from flavines. Dimerization deforms CaM binding site, for eNOS and nNOS Ca^{2+} is required for CaM binding to dimer, however for iNOS CaM can bind to dimer in the absence of Ca^{2+}. Sufficient substrate L-arg and cofactor BH_4 existence, coupling Hemes of both domains occurs and reduction of O_2 to NO proceeds.[13]

1.4 Neurodegenerative Diseases and nNOS inhibition

Over 600 diseases are linked to progressive and irreversible deteriorations occur in nervous system. Well-known diseases that occur due to these types of deteriorations are Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD) and amyotrophic lateral sclerosis (ALS). It is reported that all facts for these kinds of diseases are far beyond than single gene or multiple genes mutations or deteriorations. There are many facts that have been reported so far such as unknown and known signaling cascades, protein misfolding, protein aggregation, mitochondrial dysfunction and oxidative stress.

Silverman and his colleagues have a perspective through their recent researches and they reported in the perspective that there are five major target- and mechanism- based ways of therapy for neurodegenerative diseases; inhibition of *N*-methyl-D-aspartic acid (NMDA) receptors, voltage gated calcium channels (VGCCs) inhibition, inhibition of nNOS, Antioxidants and protein aggregation inhibition. [14] NMDA receptors are voltage gated Ca^{2+} channels that are responsible for calcium influx. With this influx, many receptors and enzymes, such as nNOS, eNOS are activated. Ca^{2+} bounded- calmodulin binds to NOS enzymes and activates these mainly eNOS and nNOS isoforms. As it is mentioned before, nNOS-derived NO molecule plays multiple crucial roles in physiological activities in nervous systems such as neurotransmitter uptake/release, neurodevelopment, synaptic plasticity. Apart from these important physiological roles, excess production of nNOS-derived NO may lead to many disorders through several chemical reactions. First of all, NO can form superoxide and by reacting with this superoxide, forms peroxynitrite ($ONOO^-$). Peroxynitrite directly nitrates tyrosines found in proteins to form nitrotyrosines therefore nitrated-proteins occur. Aggregations of these nitrated structural proteins are found in many patients suffering neurodegenerative diseases like Alzheimer's and Parkinson's diseases so these nitrated proteins can be taken as markers for nitrosative stress. [8,14]

In Alzheimer's disease, it is reported that GAPDH protein undergoes oxidative modification in the existence of excess NO and irreversibly damage glyceraldehyde-3-phosphate dehydrogenase (GAPDH) enzymatic activity. And this damage results in production of NAPDH and this product consequently damage glucose metabolism in cells. In addition GAPDH by the help of NO binds to the mitochondrial voltage-dependent anion channel protein (VDAC-1) and force it interact with neurodegeneration-related proteins and activates them. In Parkinson's disease, it has been shown that parkin and E3-ubiquitin ligase undergo same kind of NO-mediated-S-nitrosylation (S-NO). As a result of S-NO of enzymes and proteins, they impair their function and toxic protein aggregation occurs. And just like these proteins, matrix metalloproteinase 9 (MMP9), parkin, protein-disulphide isomerase (PDI) undergo NO-mediated-S-nitrosylation in the existence of excess NO and become neurotoxic through different pathways (**Figure 7**).[8]

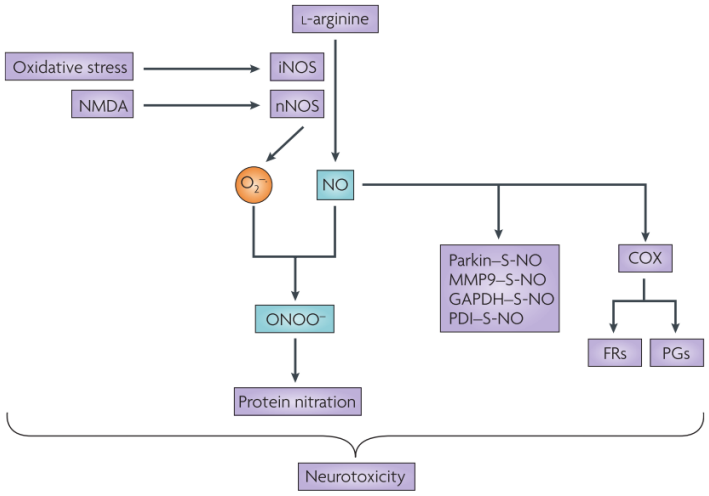


Figure 7 | **Neurotoxic Effects of Nitric Oxide.**[8]

To conclude, overproduction of NO by nNOS isoform has crucial impacts on neuronal death, impairing functions of important proteins in nervous system. It can be said that inhibition of nNOS is one of major potential therapies for many neurodegenerative diseases; AD, PD, HD, ALS. On the other hand, other isoforms of NOS enzymes should not be inhibited since they also have crucial physiological roles in endothelial cells and in macrophagal cells. Selective inhibitors for nNOS should be achieved for best, promising performance of therapeutics. Main problem is how these selective inhibitors can be achieved since there is a huge homology between three isoforms.

1.5 Drug Design and *In Silico* Approach

Designing new drug candidates and validation of this candidate is a time- and money-consuming, exhaustive process. During drug discovery and development much or less 75% of total process cost is consumed due to failures. More importantly, approximately 10 to 15 years of hard work may result as failure or success (**Figure 8**). In **Figure 8**, process of drug discovery and drug improvement is summarized step by step by showing important facts involved in each phase. Before any potential drug discovery, researchers from universities, associations and governments carry lots of projects, studies about the disease. Underlying conditions, signaling cascades, genes encoding for proteins that is involved in these cascades should be discovered to build the idea. It will take many years to turn what causes the disease into the idea of treatment of diseases. If it is resulted in success then new process, therapeutic discovery may begin. Treatments of almost all diseases for patients depend on effective therapeutics. First of all, after finding a target, a gene or protein, the target is validated to confirm relatedness of it with illness and to pre-see possible side effects. And after target validation, lead compound for that target molecule should be discovered.

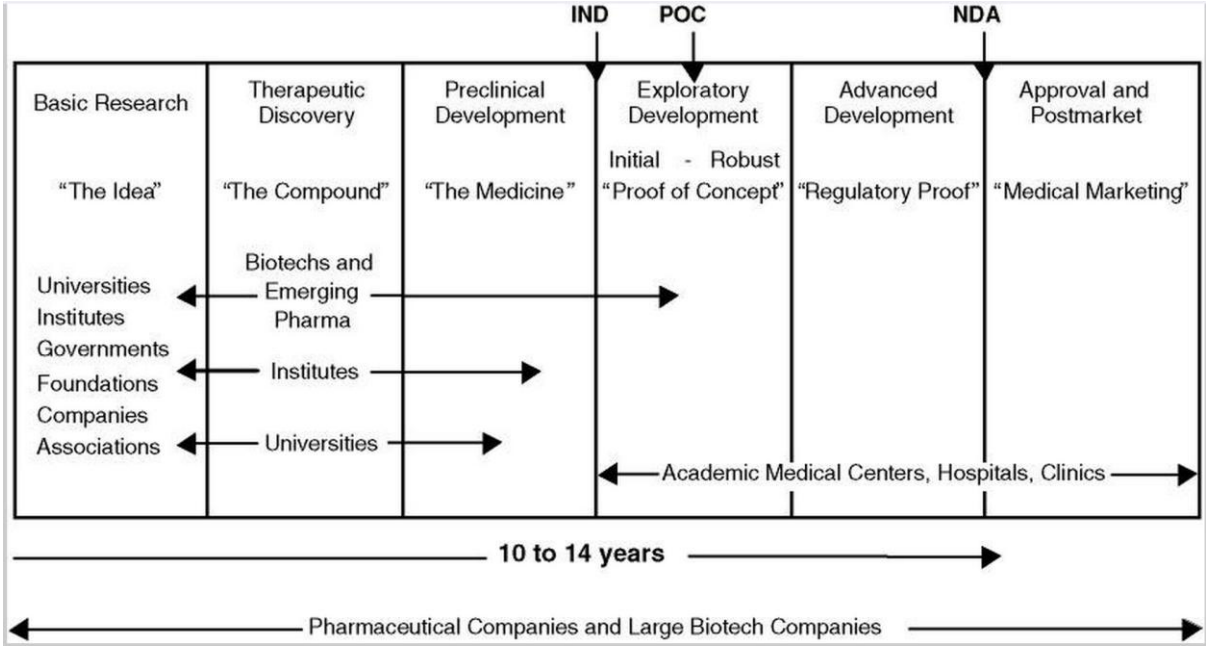


Figure 8 | **Biomedical Research from Idea to Market**. IND: Investigational New Drug application; POC = proof of concept; NDA: New Drug Application; Pharma: pharmaceutical companies.[15]

There are few ways to obtain this lead compound; nature, *de novo*, biotechnology and high-throughput screening. High-throughput screening is very fashionable way, which is used to find lead structure using compound libraries. In this approach, power of computers and robotics make possible to test and screen thousands of lead candidates and to choose promising ones from these compound libraries by docking and evaluating binding constants and investigating how lead structures link with target protein. [16]

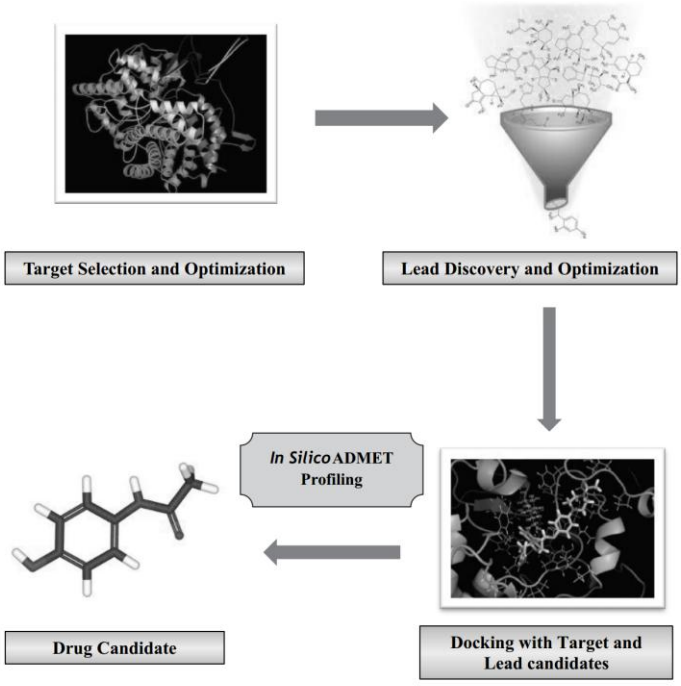


Figure 9 | ***In Silico*** **Approach during Drug Discovery Process**.[16]

In silico drug designing is another demanding approach which applies computational methods for molecular modelling.[16] In **Figure 9**, three major steps of *in silico* approach in drug discovery are summarized. There are two methods for lead generation; ligand-based and structure-based. If only knowledge is about ligands previously defined ligands as active or as inactive for target enzyme, unknown models can be aligned to these known ligands and then lead generation can be achieved. In this study, we applied structure-based *in silico* methods. To use structure-based *in silico* approach, protein-ligand interactions, structure of receptor protein must be at hand.[17] These three-dimensional receptor structures have been determined by NMR or X-Ray crystallography. And these structures are collected in

a wide library, which is known as Protein Data Bank (PDB). Today, this data bank contains almost 100 000 structures.

Virtual screening (VS) and *de novo* design are two paths used in structure-based *in silico* method. VS is a version of HTS and in some sources it can be seen abbreviated as vHTS (Virtual High Throughput Screening).[18] Molecular docking is main method in virtual screening. There are several VS tools commercially available. Some popular VS tools can be seen in **Table 1**. [19] Using these tools, drug candidates are tested by predicting compatibility of ligands with target protein. Using these predictions, 3D pose of docked ligand in the active site and binding energy of ligands can be calculated. These tools generate many representations, which can be varied by conformations, positions and orientations, of a small molecule and places each representation in the active site of receptor protein. This process aims to find most energetically favorable pose of a small molecule in the active site of receptor. To calculate pose predictions from many binding modes of small molecule and affinity predictions of best posed small molecules, various algorithms and scoring functions have been developed and applied by different docking programs. [17,20]

TOOL	VENDOR
DOCK	University of California at San Francisco
Gold	Cambridge Crytallographic Data Centre
AutoDock	The Scripps Research Institute
FlexX	Tripos, Inc
Glide	Schrödinger, Inc
ICM	Molsoft, Inc
LigandFit	Accelrys, Inc

Table 1 | **List of Some Commercially Available VS Tools and Their Vendors**. [19]

After obtaining promising candidates, they should go through for several tests to assess early safety of the lead compound. In this step, early tests for safety, efficacy and potential toxicity of drugs are done. These studies are also needed for researchers to propose Phase I studies. In Phase I, scientists apply some tests considering several important properties to obtain important information about drug's properties. In which dose drug shows successful impact on therapy with minimizing its possible side effects? How good are drug's pharmacokinetic properties and toxicity? Does drug show potential effects on other biological molecules rather than its target molecule? Are effects of drug candidate influenced by another drug? Such queries should be answered at this phase. To come with pharmacokinetic properties of drug (ADME) and its toxicity (Tox), a successful drug must be absorbed in bloodstream (**A**bsorption), distributed to target site of molecule in the body (**D**istribution), metabolized in an efficient and an effective way

(**M**etabolism), excreted successfully from the body (**E**xcretion), not show toxicity in the body (**T**oxicity). And these ADME/Tox studies can be performed in living cells, in animals and also by applying computational tools. Phase I is the first phase in which drug can be tested in human in a small group of healthy volunteers. So this phase is one of the crucial phases during drug discovery process. [15]

Second phase clinical trial is done on a small group of patients. In Phase II, researchers try to obtain information about drug's effectiveness on patients with illness and to examine adverse events and risks showed by drug. After these examinations, if the drug is found promising, these almost same examinations are tested in large group of patients as Phase III clinical trials. It is crucial to do these trials as large as and as diverse as possible group of patients. So many clinics, regions are involved in this phase. After completing and being successful in all three phases, New Drug Application (NDA) must be written and FDA approval must be received. [15]

1.6 Selective Inhibition of Neuronal Nitric Oxide Synthase

In mammalian cells, nitric oxide produced by nitric oxide synthases has crucial roles during several physiological and pathophysiological processes as they are mentioned before. Since there are three isoforms of NOS and they are expressed in

different tissues, each NOS isoforms produce NO for separate biological processes in human body. In addition, we mentioned before that overproduction of NO by nNOS is mainly related to many neurodegenerative diseases, chronic headache, stroke, Alzheimer's, Parkinson's, Huntington's diseases. [4 21] Therefore, achieving selective nNOS inhibition in the brain without inhibiting eNOS and iNOS is important focus as therapeutic for neurodegenerative diseases. On the other hand, that there is a approximately 50% sequence homology and high similarity in heme active site of all three isoforms of NOS makes this task very difficult. [22] Residue differentiation between isoforms in their active sites may be one of major facts that should be maintained on the way of achieving selectivity. Aligning three isoforms (**Figure 3**, **Figure 4**) reveals these differences as following: S585 in nNOS is N370 in iNOS; D597 in nNOS is N368 in eNOS. [14] Some active site residues in three isoforms and differences among these residues are summarized in **Table 2**.

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

Table 2 | **Some Residues in Three Isoforms' Active Sites**. Residue differences are indicated in bold.

To conclude, selective inhibition of nNOS in the brain is crucial task considering neurodegenerative diseases, therefore, many researchers focus on to discover good selective nNOS inhibitors. On the other hand, due to mentioned similarity of three isoforms, these researches are exhaustive challenges. That's why, no drug, which serves for this purpose as good potential and selective inhibitor has yet been designed. [6]

2 MATERIALS AND METHODS

2.1 Ligand and Enzyme Preparations

Three isoforms of nitric oxide synthases were collected from Research

Collaboratory for Structural Bioinformatics (RSCB) protein databank. Among all NOS PDB crystal structures, 1OM4 (nNOS with L-arginine bound, resolution 1.75Å), 3DQS (eNOS with inhibitor C_{20} H_{28} Cl N_5, resolution 2.03Å), and 1NSI (iNOS with L-arginine bound resolution 2.55Å) were used for all analysis.

For enzyme preparation, except chain A of all structures, all other chains and all solvent molecules exist in PDB structures were eliminated. However, heme group and H_4B were left in the active sites of structures since these cofactors should be involved in energy calculations during docking. Protein Preparation protocol and then "Clean Geometry" toolkit included in Discovery Studio 3.1 software package (Accelrys, Inc.) were employed to do energy minimizations and preparations of enzymes to make them dockable. 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. Inhibitors designed by Richard B. Silverman [Richard B. Silverman, March 2013] [Richard B. Silverman, June 2013] and his colleagues and newly designed ligands were drawn and prepared *in silico* using Discovery Studio 3.1. "Clean Geometry" toolkit is also used to prepare and minimize drawn ligands.

2.2 Generation of Potential Inhibitors

2.2.1 Test Inhibitors

Richard B. Silverman and his colleagues designed and synthesized inhibitors for selective inhibition of nNOS. [6, 22] To control our *in silico* docking calculations, we prepared these ligands on Discovery Studio, can be seen in **Figure 10**, designed by Silverman and they are docked into each isoforms of NOS selected by us. And we compared experimentally calculated inhibition constants with our computationally calculated results.

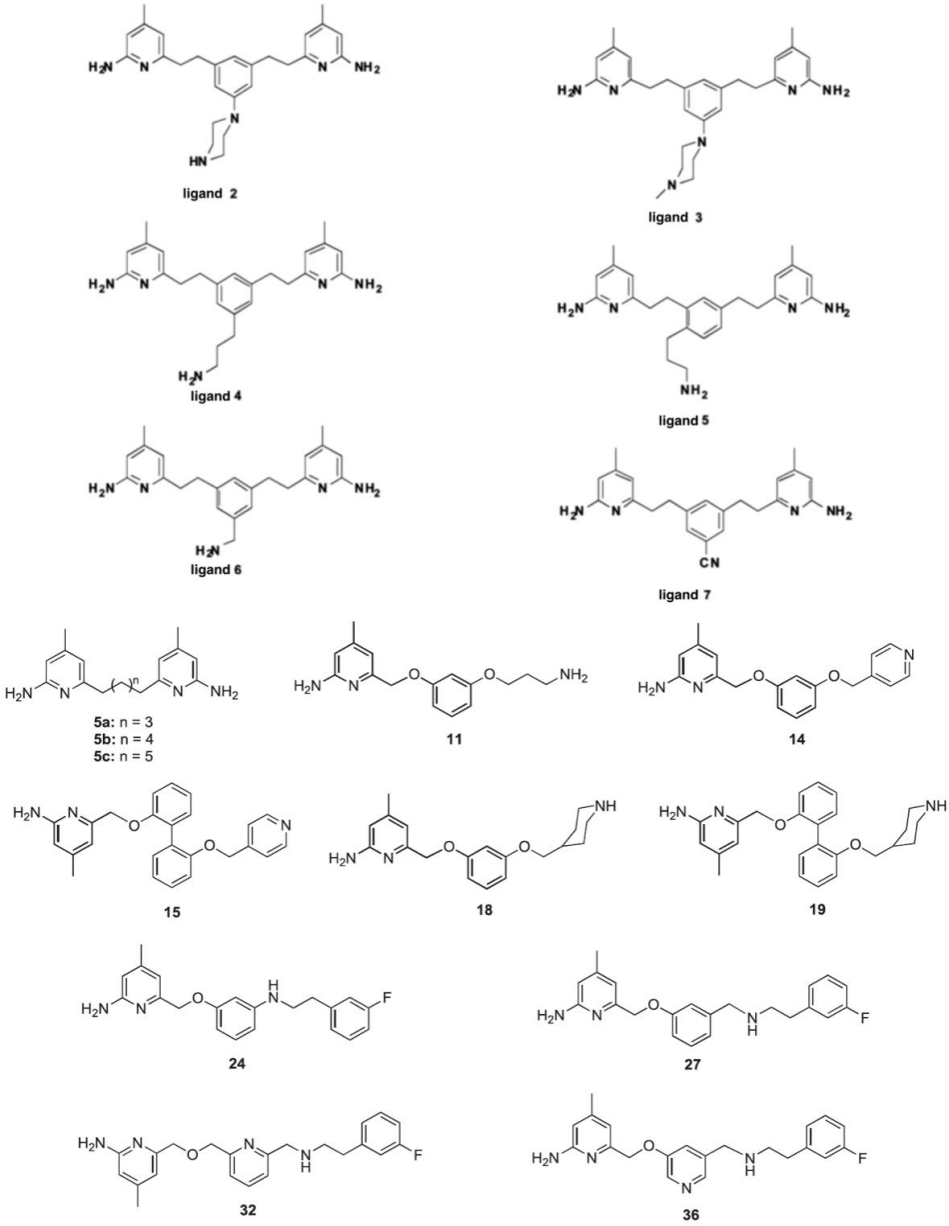


Figure 10 **| Designed and Synthesized Ligands by Richard B. Silverman**. [6, 22]

2.2.2 Inhibitor Candidates

Two scaffolds (**Figure 11 and 12**) were created and used for derivation of our inhibitor candidates. **Table 3** and **Table 4** show two groups of potential inhibitors derived from two separate scaffolds.

GROUP A

Lead Scaffold

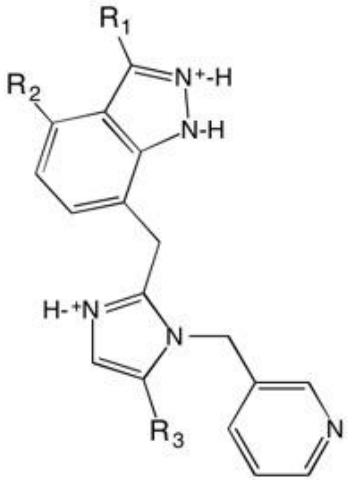
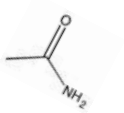
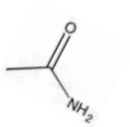
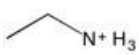
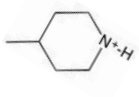


Figure 11 | **First Scaffold Used in this Study.**

Designed Inhibitor Candidates	**R_1**	**R_2**	**R_3**
KB20		-H	-H
KB21		-H	-H
KB22		-F	-H
KB23		-F	-H
KB24		-F	
KB25		-F	

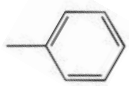
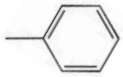
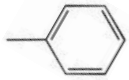
KB26		-F	
KB27		-H	
KB28		-F	-H
KB29		-H	-H
KB30		-H	

Table 3 | **Lead IDs of First Group of Potential Inhibitors and Corresponding Functional Groups.**

GROUP B

Lead Scaffold

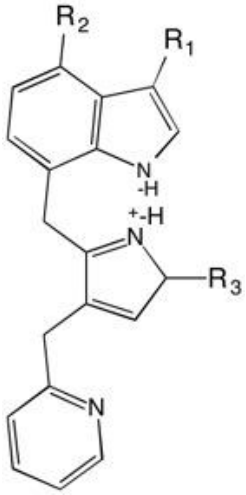


Figure 12 | **Second Scaffold Used in this study**

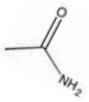
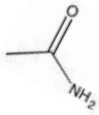
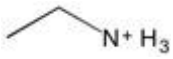
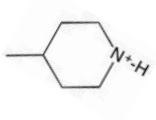
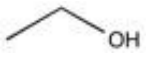
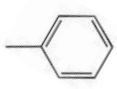
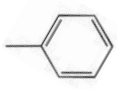
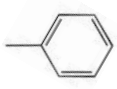
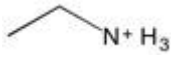
Designed Inhibitor Candidates	R_1	R_2	R_3
KB31		-H	-H
KB32		-H	-H
KB33		-F	-H
KB34		-F	-H
KB35		-F	
KB36		-F	
KB37		-F	
KB38		-H	
KB39		-F	-H
KB40		-H	-H
KB41		-H	

Table 4 | **Lead IDs of Second Group of Potential Inhibitors and Corresponding Functional Groups.**

2.3 Docking

Binding affinities and docking orientations of inhibitor candidates were calculated applying famous docking virtual tool, AutoDock. Predictions of binding affinities of flexible ligands into target enzymes, in this project targets are eNOS, iNOS and nNOS, are obtained via using AutoDock 4.2. For calculations of these binding affinities, AutoDock applies empirical binding free energy function based on AMBER force field. [23] As conformational search method, AutoDock uses Lamarckian genetic algorithm. AutoDock Tool (ADT) was used to set up AutoGrid parameter files (gpf) and AutoDock parameter files (dpf) which include required parameters during docking simulations. Beforehand, the charge of Fe atom of heme in all three enzymes was changed from +2 to +3. The ligands were docked inside a grid box with 60 A^o x 60 A^o x 60 A^o dimensions and grid spacing 0.375 A^o. For center of the grid box, centers of ligands found in original PDB structures' active sites were noted and used.

Docking simulations were performed applying Lamarckian genetic algorithm. Genetic Algorithm Parameters were set to 10 independent LGA runs, 150 in population size, 5000000 in energy evaluations and 27000 in generations and all other parameters were remained as default.

Inhibition binding constants (K_i) of best runs in each resulting docking

orientations were collected for further analysis.

3 RESULTS AND DISCUSSION

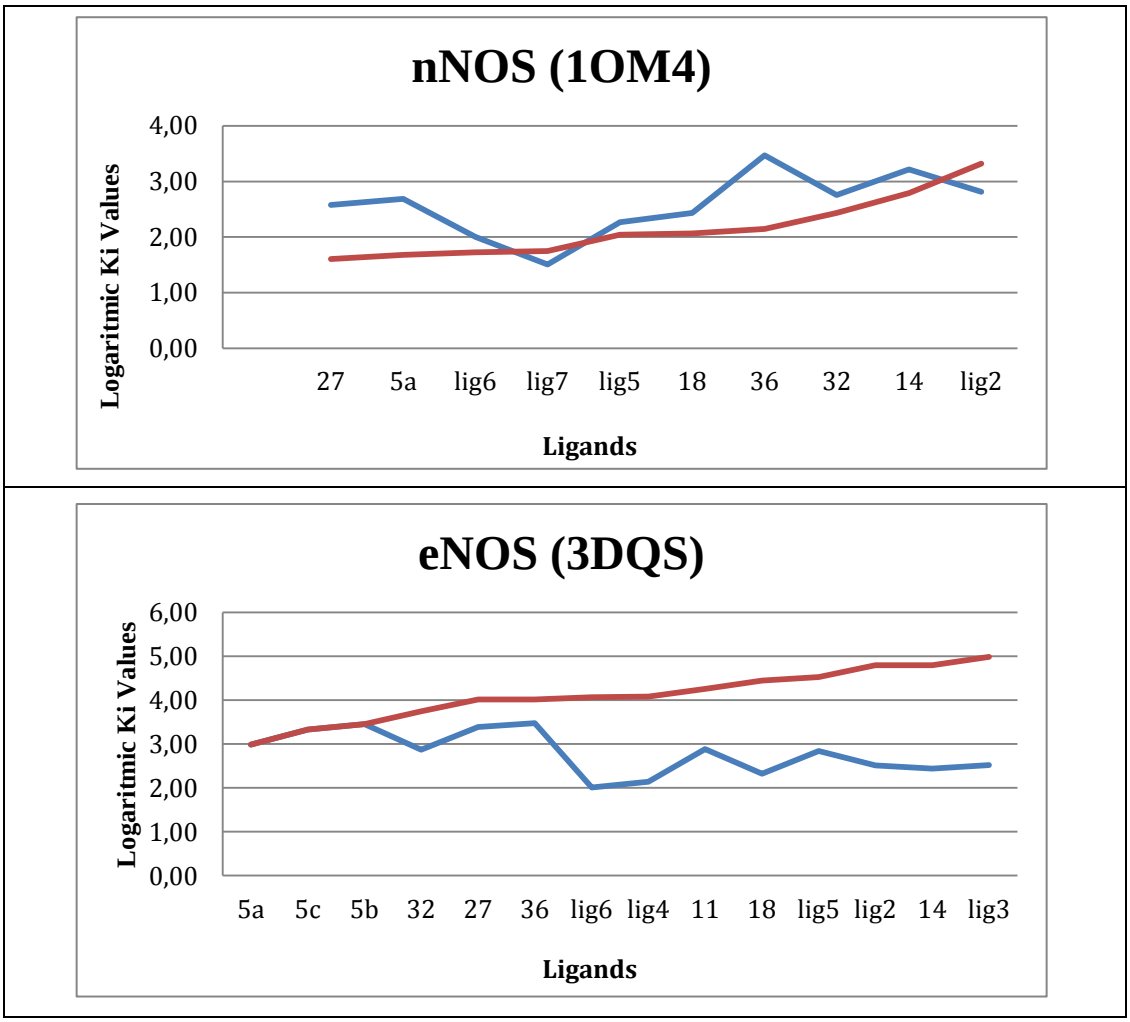
During enzyme inhibition, if any drug can work at low concentration and do not inhibit or affect other enzymes at this concentration can be counted as strong selective inhibitors. In this study, our aim is to obtain this kind of potential strong selective inhibitors for nNOS enzyme applying computational tools. There are other physiologically important isoforms of NOS enzyme, eNOS and iNOS, and high homology in the active sites of these three isoforms makes our task challenging. (**Figure 3**, **Figure 4**). Therefore, selectivity of potential inhibitors between three isoforms becomes main challenge to be considered.

In previous projects held by Prof. Kemal Yelekçi's group, all PDB structures account for three isoforms of NOS enzyme were used for comparative validation studies to select most reliable PDB structures of NOS isoforms to be used in further projects. In that project, studies such as computational modeling and re-docking of bound ligands in crystal structures were applied. Also some properties such as resolutions of PDB structures, RMSD values from re-docking simulations to compare with original co-crystallized position of ligands within active site were

considered to choose most compatible PDB structures among all. As a result, PDB structures with code 1OM4 for nNOS, 3DQS for eNOS and 1NSI for iNOS were selected to be used in all docking simulations. [24] Relying on these results, we also focused and used these three structures for all docking simulations.

At the most beginning, inhibitors designed by Silverman (**Figure 10**) were drawn by us on Discovery Studio and re-docked to the prepared enzymes by us and binding modes were calculated. X-Ray structures of NOS enzymes with bound inhibitors designed by Silverman, which are placed on publication of Silverman, were downloaded from PDB website. Those binding modes were compared with already originally bound poses in downloaded structures. Ultimately, we observed close binding modes in the active site of all isoforms. Additionally, inhibition constants obtained from these re-docking results were checked against inhibitor constants experimentally obtained by Silverman group. To compare these computationally and experimentally obtained inhibitor values, all constants were converted to nanomolar units and their logarithmic values were calculated. These logarithmic values of experimentally and computationally obtained inhibition constants were plotted for each isoform (**Figure 13**). By re-docking already synthesized inhibitors, we aimed to crosscheck reliability of our virtual screening results and grid and docking parameters, which are considered in our docking

simulations.[6] To comment **Figure 13**, behaviors of experimental and computational plot lines are very close to each other. In fact, many results have given almost same values with experimental values that can be seen as interceptions in plot lines. Since there are many facts that can affect and change docking and scoring processes during *in silico* screening, we cannot wait to obtain exact same values. These results obtained from our control studies are enough to fulfill our expectations.



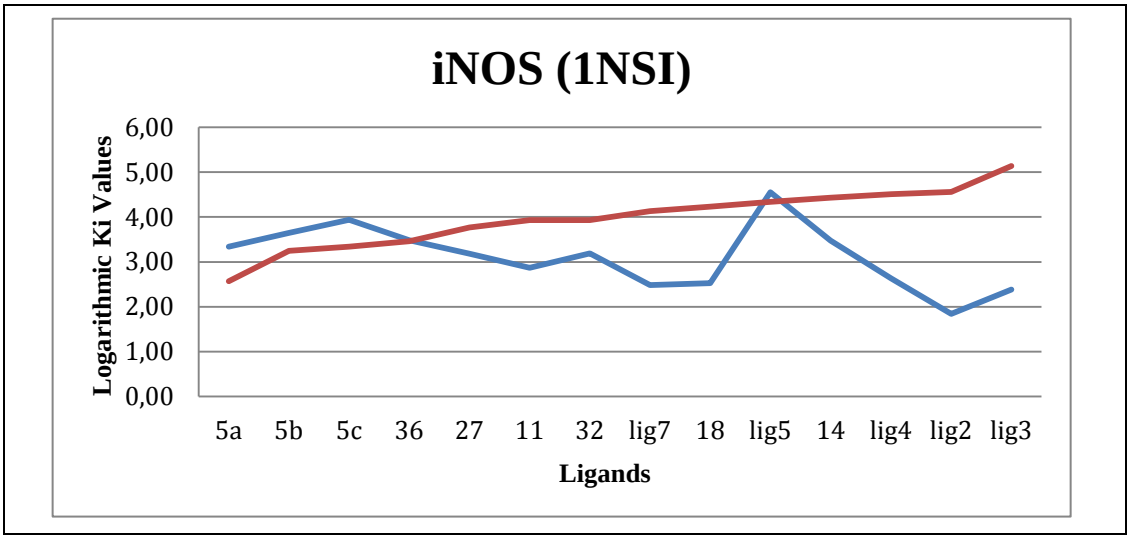
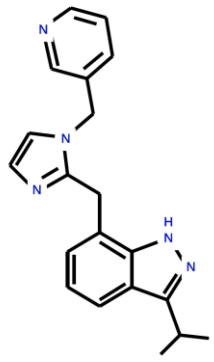


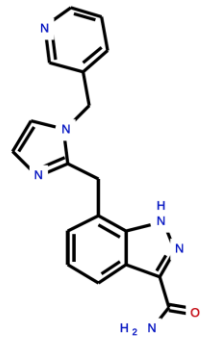
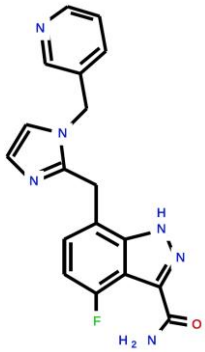
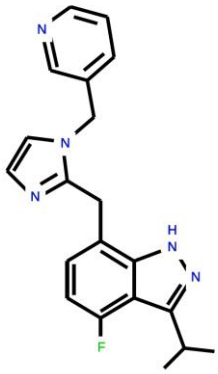
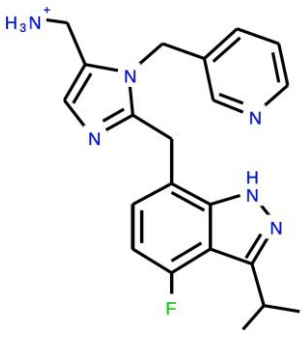
Figure 13 | **Plots of Experimentally and Computationally Obtained Inhibition Constants Values (K_i).** Docking experimentally synthesized ligands within active sites of three isoforms resulted inhibition constants (K_i). These plots represent log values of these computationally obtained K_is and experimentally obtained K_i s for comparison analysis. Blue lines represent computational values and red lines represent experimental values.

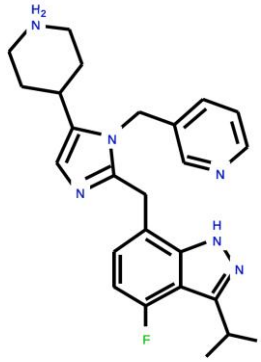
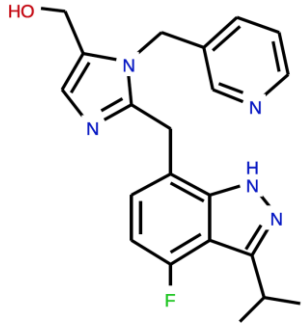
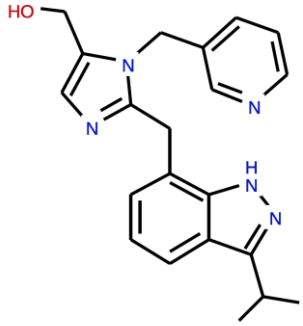
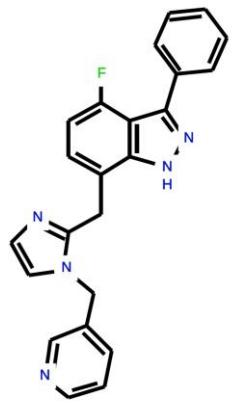
In **Figure 11** and **12**, two scaffolds that were used in this study were presented. Structure-based drug design is applied to obtain these scaffolds and leads. More than several hundred lead compounds in the ZINCv12 lead library considering their structural and physicochemical properties, which selectively inhibit nNOS isoform, were scanned. [25] In previous projects, by utilizing ZINC and Accelrys 3.1 fragment-based libraries, which contain about hundred thousand fragments, about 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 method. After docking all analogues into three isoforms of NOS, only inhibitors that selectively inhibit nNOS were used for further modifications. [24] The

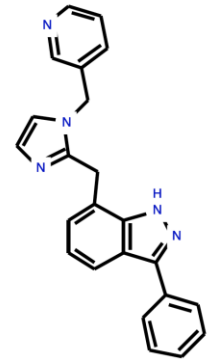
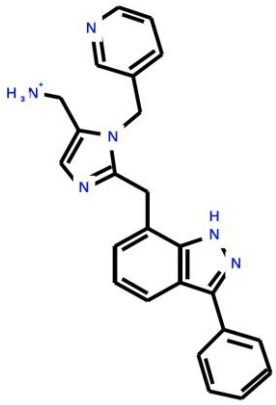
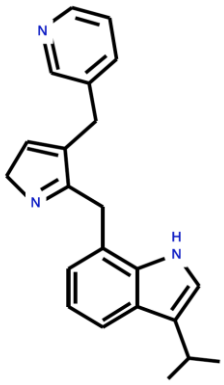
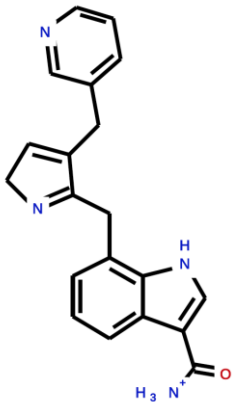
structure-based methods were employed manually for the further optimization of the potential nNOS inhibitors by adding and removing a few fragments on the scaffolds. 22 potential inhibitors (**Table 3** and **4**) among all inhibitors for nNOS selective inhibition is designed using mentioned methods on two scaffolds. These 22 derived leads from two scaffolds were selected to discuss on them. Current designs and computational evaluations of these 22 potential inhibitors using various docking tools are listed in

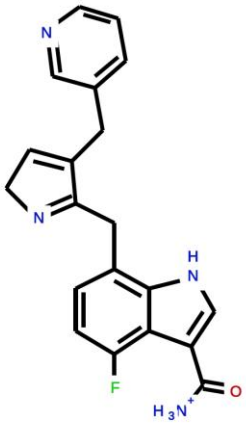
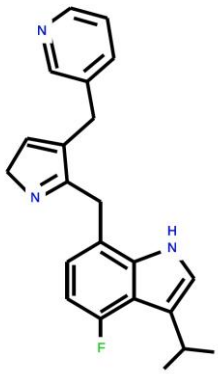
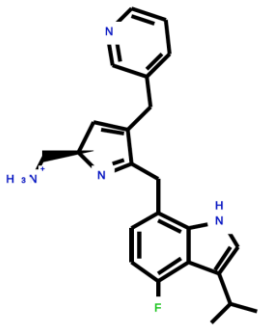
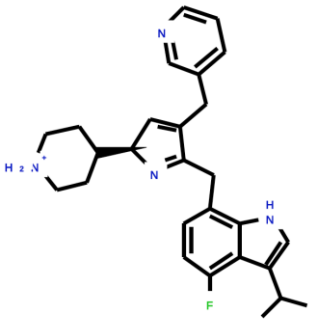
Table 5.

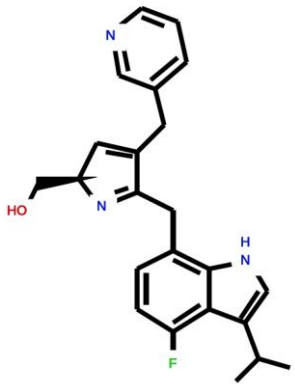
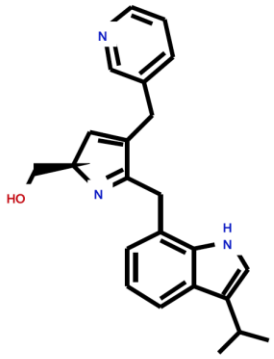
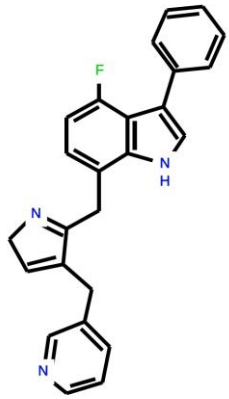
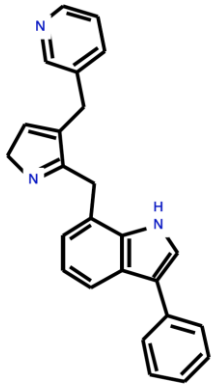
Inhibitors	**Chemical Structures**	**nNOS (1OM4)**	**eNOS (3DQS)**	**iNOS (1NSI)**
Kb20		2730	6710	6600

Kb21		872	11830	1680
Kb22		1930	36770	2120
Kb23		1640	13670	3550
Kb24		202	8640	277

Kb25		1470	30190	372
Kb26		1250	2320	1200
Kb27		949	1760	1200
Kb28		746	2840	659

Kb29		506	1730	324
Kb30		606	1380	74
Kb31		1000	3180	816
Kb32		245	5580	572

Kb33		550	3260	1500
Kb34		589	5150	1730
Kb35		75	11010	594
Kb36		97	211	3840

Kb37		365	1650	857
Kb38		291	1090	490
Kb39		203	971	241
Kb40		192	639	314

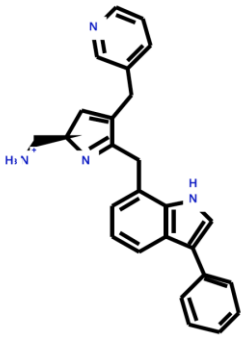
Kb41		15	470	31

Table 5 | **Chemical Structures of the 22 Designed Potential NOS Inhibitors and Their Inhibition Values Obtained from Docking Simulations.** All inhibition values are given in nanomolar concentration.

Considering derivations of two scaffolds and docking results based on inhibition constants (K_i) present in **Table 5**, we can discuss many facts for nNOS selective inhibition. Referring first scaffold, results and derivations may reveal several facts. Between Kb20 and Kb21, there is one functional group differentiation. In Kb20, isopropyl is replaced with propanamide. It can be easily seen that this functional group differentiation leads increase in affinity of inhibitor for nNOS. In addition, selectivity of nNOS over eNOS also increased, however, selectivity of nNOS over iNOS decreased. Fluoro derivative of Kb21 is Kb22. eNOS inhibition value increased three-fold, inhibition value of nNOS is doubled whereas value for iNOS is slightly increased.

Comparisons between Kb20 and Kb23 and between Kb21 and Kb22 show that addition of fluorine has good impact on selectivity of nNOS over eNOS.

Additional functional amine group to Kb23 resulted in stronger inhibition of Kb24 on all three isoforms but selectivity of nNOS isoform over iNOS is dramatically decreased. And another group addition to Kb23 instead of amine group drastically increased eNOS inhibition value but undesirably decreased iNOS inhibition constant.

Using alcohol group as a functional group in the first scaffold does not provide well for our aim of nNOS selectivity since it reduces K_i for eNOS and approximates it to K_i of nNOS. In Kb26 and Kb27, slight differences in inhibition values of all isoforms can be seen. These results do not meet expectations of us.

For Kb28, Kb29 and Kb30, benzene group is preferred as one of major functional group in lead scaffold. Based on results for these three inhibitors, it can be said that benzene group in the first scaffold does not provide the desired selectivity order, since inhibition constants are close to each other and both inhibitors selectively inhibits iNOS rather than nNOS.

In the second scaffold for functional group R_1, it is acquired that using propanamide group instead of isopropyl group serves better for our purpose. There is no selectivity of nNOS over iNOS for inhibitor Kb31. However Inhibitor Kb32 achieved two-fold selectivity and reduced nanomolar concentration for inhibition of nNOS.

Another interesting and promising discrimination was observed with a shift between Kb34 and Kb35. Between these two inhibitors, only R_3 group was changed from hydrogen to 1-amino methyl group. This change resulted in dramatic selectivity of nNOS over eNOS and of nNOS over iNOS.

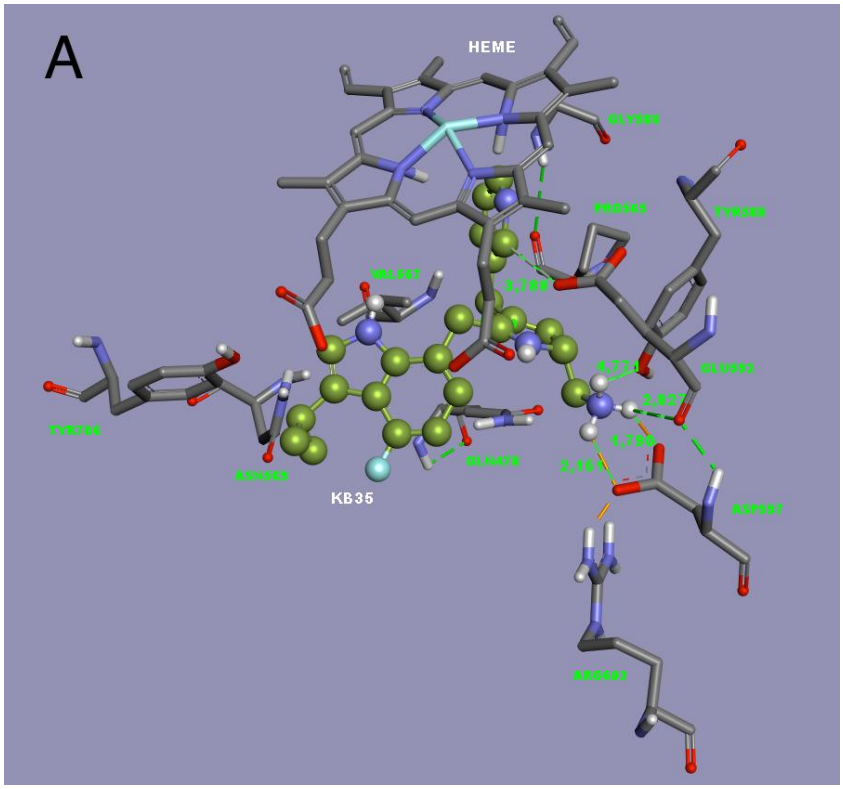
Replacing 1-amino methyl in Kb35 with pyridine, Kb36 is obtained. This shift causes obvious decline in inhibition value for eNOS. In Kb36, better selectivity of nNOS over iNOS is achieved, but good selectivity of nNOS over eNOS observed in Kb35 was lost. eNOS inhibition reached the strongest inhibition comparing other candidates.

When alcohol group is used for R_3 functional group instead of hydrogen, slight stronger inhibitions were observed for all isoforms (Kb34, Kb37). Eliminating F atom from Kb37 structure, more decline in inhibition values of isoforms obtained in docking simulations of Kb38.

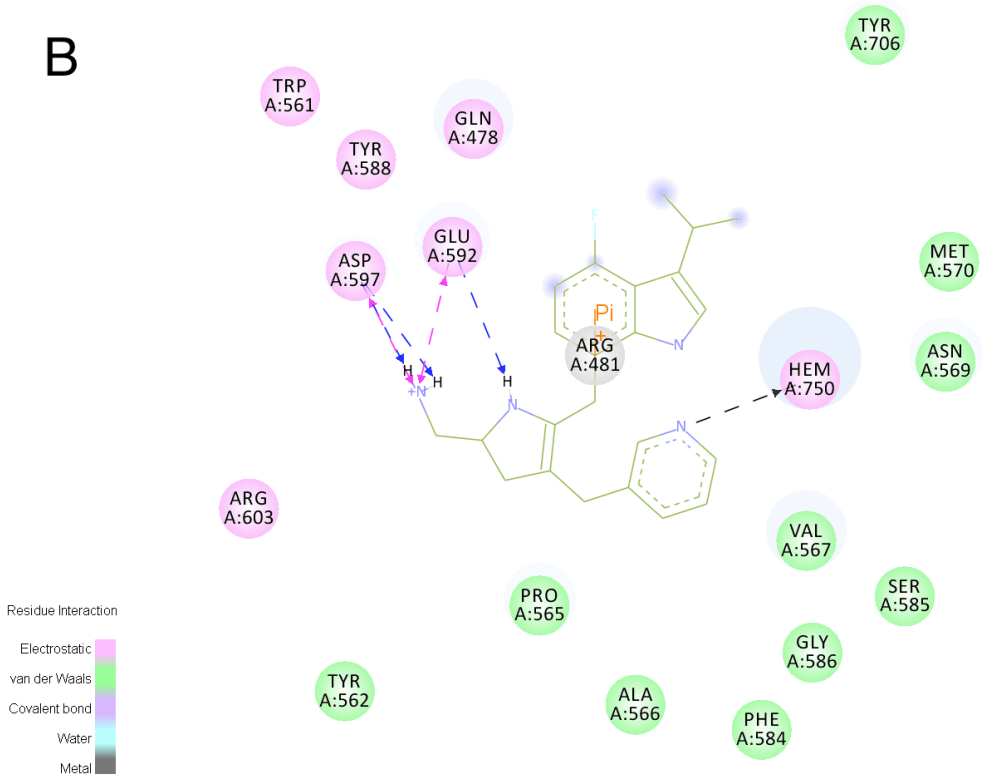
Benzene ring is majored as functional group R_1 for Kb39, Kb40 and Kb41. In these three inhibitor candidates, docking calculations resulted in close inhibition constants for iNOS and nNOS, so selectivity of nNOS over iNOS is almost lost. Considering derivatives with benzene ring in Group A and B, it can be concluded as using benzene ring as a functional group in first and second scaffold leads stronger inhibitors for eNOS.

Docking simulation results of all candidates used in this study highlighted some important facts on the way of obtaining the most promising inhibitor. In all 22 designed candidates it can be seen that nNOS selectivity over eNOS is achieved with more inhibitors than nNOS selectivity over iNOS. Almost all candidates bind iNOS more tightly than eNOS. Substitution of benzopyrazole and imidazole ring in scaffold one with benzopyrrole ring and pyrrole is increased nNOS selectivity and potency as it can be clearly seen if results of group A candidates and group B candidates are compared. Attaching 1-amino methyl group to pyrrole ring made us to obtain best nNOS selectivity over iNOS. Inhibitor Kb35 binds more tightly to nNOS among all isoforms. Selectivity of nNOS over eNOS (selectivity ratio nNOS/eNOS) is 146 fold and selectivity of nNOS over iNOS (ratio nNOS/iNOS) is 8 fold. Removing 1-amino methyl group from pyrrole ring, in Kb34, lead decrease in both selectivity and potency of nNOS. Subsititution of 1-amino methyl with pyridine ring attached to pyrrole ring, compound Kb36, made us to obtain best selectivity of nNOS over iNOS, which is 39 however selectivity of nNOS over eNOS, which is 2 in Kb36 and 8 in Kb35, is lost.

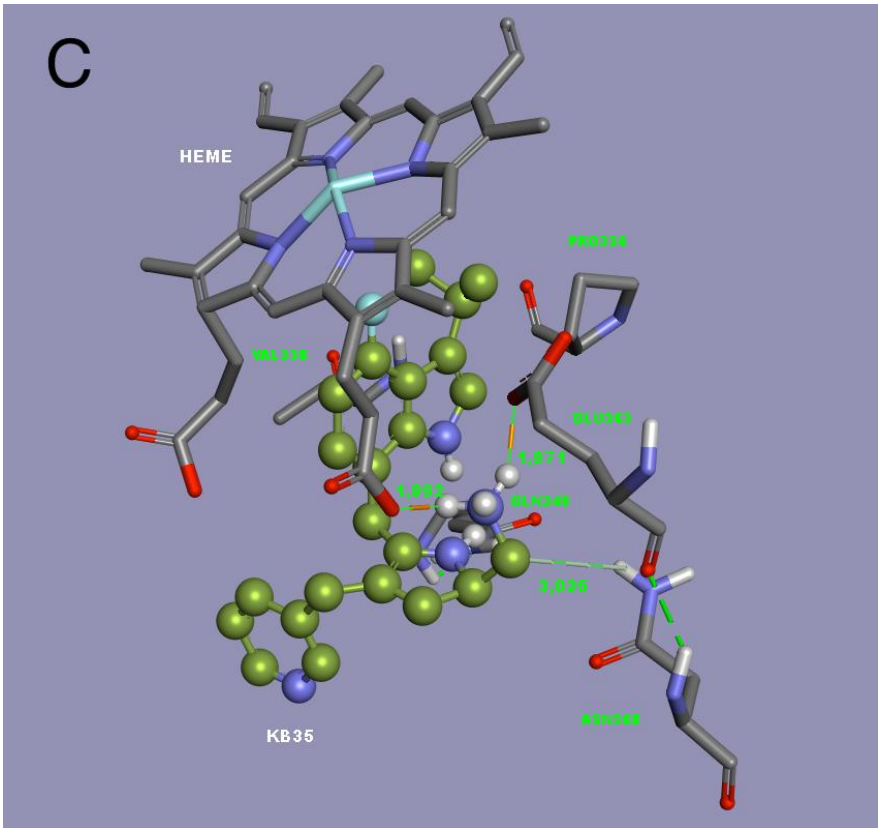
To depict detailed binding interactions of docked compounds, analysis of optimal binding modes are done with compound Kb35. Best docked poses of Kb35 and significant interactions in actives sites of all isoforms can be seen in **Figure 14.**



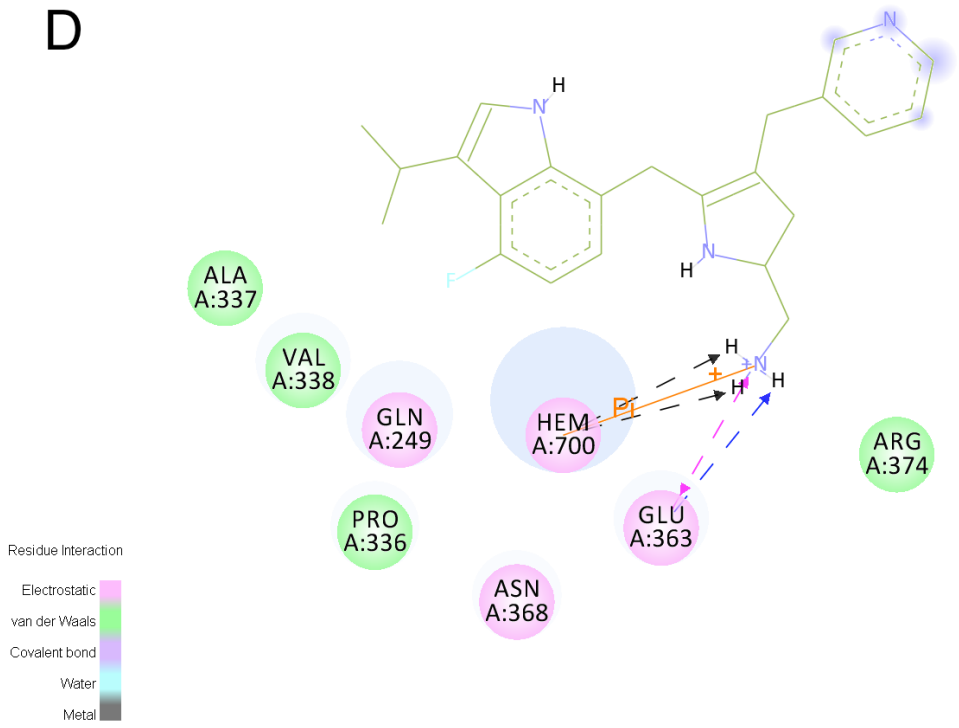
A. 3D interaction diagram of Kb35 in the active site of nNOS.



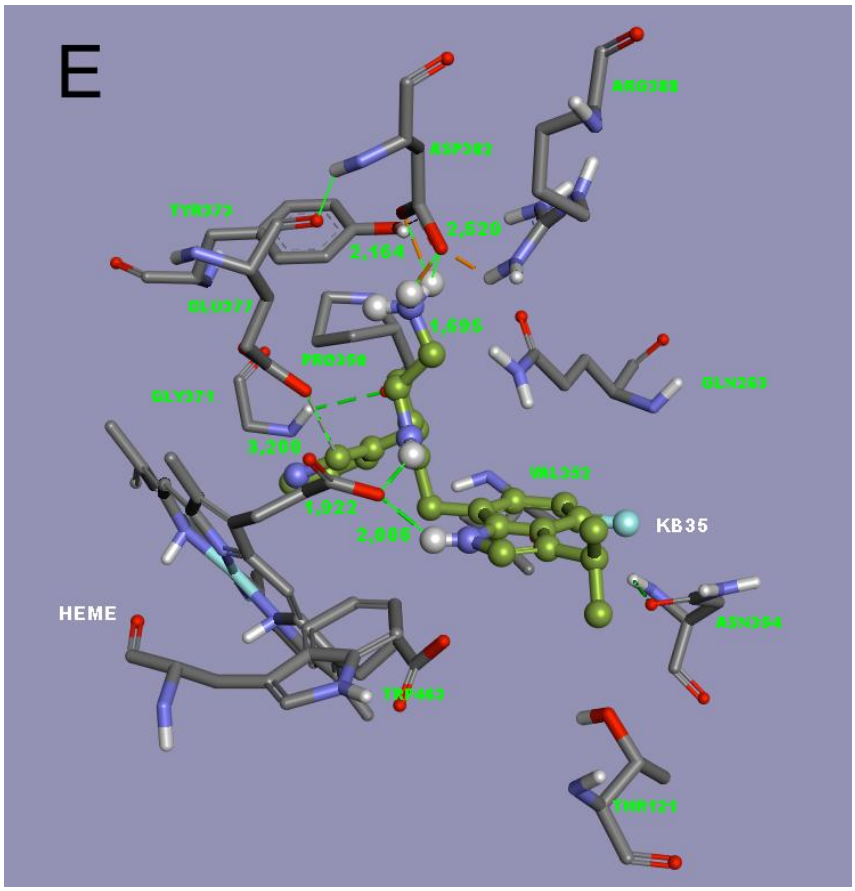
B. 2D interaction diagram of Kb35 in the active site of nNOS.



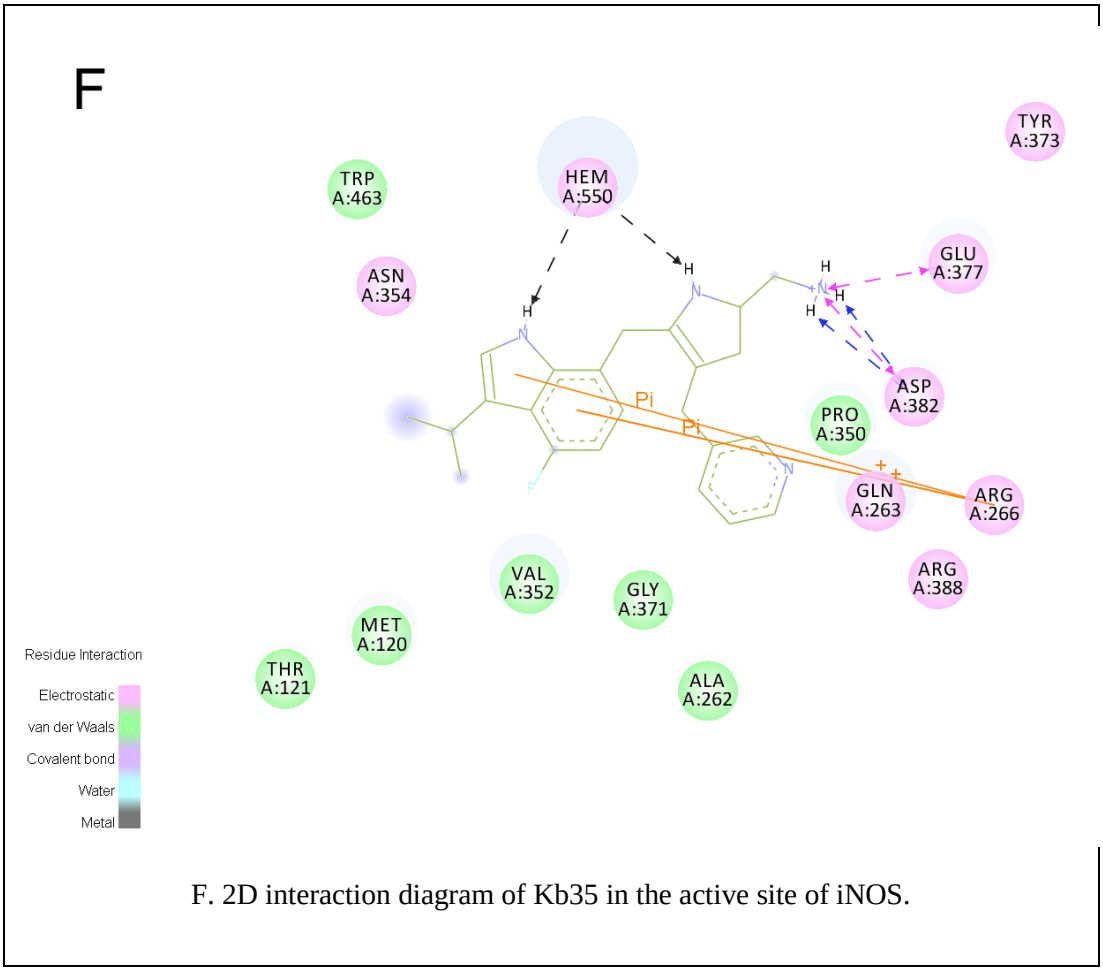
C. 3D interaction diagram of Kb35 in the active site of eNOS.



D. 2D interaction diagram of Kb35 in the active site of eNOS.



E. 3D interaction diagram of Kb35 in the active site of iNOS.



F. 2D interaction diagram of Kb35 in the active site of iNOS.

Figure 14 | **3- Dimensional and 2- Dimensional Orientation Diagrams of Best-Docked Poses of Kb35 in Active Sites of Three NOS Isoforms.** In 3D diagrams (A, C, E), amino acid side chains and heme cofactor within the volume of 3,5 Å distance from inhibitor are shown with sticks configuration. Compound Kb35 is shown with scaled ball and stick configuration. Dashed lines represents hydrogen bond interactions. In 2D diagrams (B, D, F), pink, green, purple, blue and black dashed lines represent electrostatic, van der Waals, covalent, hydrogen and metallic interactions, respectively.

Optimal binding analysis shows that best poses of Kb35 docked in all isoforms are located in the vicinity of cofactor. (**Figure 14)** Predicted pose of Kb35 in nNOS active site cavity interacts with some significant amino acid residues found in the cavity. (**Figure 14 A**) Two hydrogen atoms on the 1-methy amino group formed two strong hydrogen bonds with amino acid residue ASP597 (Distances are 1.790 Å and

2.15 Å) and one of them also formed H-bond with GLU592 (with 2.827 Å distance). Last hydrogen atom of 1-methyl amino group formed H-bond with TYR588. A carbon hydrogen bond is formed between carbon atom of pyrrole and carboxylate group in side chain of GLU592. Best pose of Kb35 is fitted within an active site cavity seen in **Figure 14 A** which includes GLY586, PRO565, TYR588, GLU592, ASP597, ARG603, GLN478, ASN569, TYR706 and VAL567. Polar and hydrophobic interactions are formed between Kb35 and these amino acids found in pocket. In **Figure 14 B**, nNOS isoform and Kb35 is represented with two-dimensional diagram. In this diagram, 17 amino acid residues closely interact with Kb35 via electrostatic, VDW, covalent bond interactions that are highlighted.

3D and 2D diagrams in **Figure 14 C, D** reveal intermolecular interactions between Kb35 and eNOS isoform. VAL 338, GLN 249, PRO336, ASN368, GLU363 are amino acid residues which are in close interaction with Kb35 within a vicinity of 3,5 Å distance. One hydrogen atom of 1-amino methyl group interacts with propionate side chain of heme cofactor via strong H-bond (distance is 1.892). GLU363 side chain carboxylate forms another H-bond with 1-amino methyl group. Carbon atom of this group forms two carbon H-bond with GLU363 and ASN368 (distance are 3.729 Å and 3.036 Å, respectively). These interactions are numerically less and weaker than the interactions predicted in nNOS active site cavity.

Additionally, residues closely in contact with Kb35 within eNOS active site cavity are not as many as residues found in nNOS active site cavity. All of these facts are evidential for that affinity of Kb35 to nNOS dominates affinity of Kb35 to eNOS. And this explains selectivity ratio nNOS/eNOS which is 1/146.

Figure 14 E, F are representations for interactions between Kb35 and active site residues of iNOS isoform. Active side residues interacting with Kb35 are TYR373, GLU377, ASP382, TRP363, THR121, GLN263, ASN354, VAL352, GLY371 and PRO350. Propionate oxygen atom of heme cofactor is in interaction with two hydrogens; hydrogen atom of pyrrole ring and of benzopyrrole ring (distances are 2.086 Å and 1.922 Å). Oxygen atoms in carboxylate side chain of ASP382 form tripod strong H-bonds with 2 hydrogen atoms in 1-amino methyl group (distances are 2.164 Å, 2.520 Å and 1,695 Å). Carbon atom of pyridyl ring also form a H-bond with another amino acid residue GLU 377. There are 14 amino acid residues closely interact via different bond types with Kb35 as they can be seen in **Figure 14 F**.

In all binding pose predictions of Kb35 within three isoforms, there is something interesting that attracted our attention. nNOS and iNOS isoforms contain ASP597 and ASP382 at the same place in aligned isoforms, however eNOS contains ASN368 intead of ASP (**Table2**) . In nNOS and iNOS binding pose predictions both

ASP597 and ASP382 interacted with compound Kb35 via strong hydrogen bonds, however pose predictions between eNOS and Kb35 did not reveal any strong bonds between compound Kb35 and ASN368. As it is mentioned selectivity of nNOS over eNOS is much more than selectivity of nNOS over iNOS. This fact makes us to question any possible impacts of residue differences on selectivity of inhibitor.

CONCLUSION

There is almost 50% sequence homology between three isoforms of NOS enzymes and going deeper shows high similarity in the active sites of nNOS, eNOS and iNOS. That's why, designing and developing a selective inhibitor for nNOS become very challenging task. Virtual screening tools were employed in this present project and these studies highlighted that designed and selected lead scaffolds, especially the second one could meet the expectations of us. It is proven that these lead scaffolds are important candidates for further optimization analysis and modifications to obtain promising inhibitor candidates that would be employed as potential and selective inhibitors for nNOS. Among 22 selected inhibitors, Kb35 is found as the most promising candidate. Best pose predictions and scoring methods showed that introducing 1-amino methyl group to pyrrole ring increase compound's

affinity and selectivity towards nNOS. Compound Kb34 is modified by removing 1-amino methyl group from pyrrole ring and it is clearly seen that both affinity and selectivity of compound towards nNOS declined. Designed compound Kb35 inhibits nNOS 146 fold better than eNOS and 8 fold better than iNOS. These results also showed us that subtle residue differences in active sites of isoforms could be important indicatives and determinants for selective and potential inhibitors. In this present work, promising lead scaffolds and important determinants for selective inhibition of nNOS were discovered via various computational tools and virtual screening tools. Further studies with regard to these important findings would direct researchers to design promising potential and selective nNOS inhibitors.

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Curriculum Vitae

Bahanur Örtmen was born on May 8th 1989 in Bursa. Her B.Sc. degree has been earned in Molecular Biology and Genetics in 2012 from Bilkent University. During Summer 2010 and 2011, she was accepted to work as a research assistant and an intern in Orwar Labarotary at the Department of Chemical and Biological Engineering at Chalmers University of Technology. And she assisted four projects focusing on biomembranes, liposomes and phospholipid nanotube networks during these periods. After completing her undergraduate, she was accepted to Kadir Has University to study her graduate on Computational Biology and Bioinformatics in September 2012. Multidisciplinary approach by combining computational biology and life sciences is her main interest.

Publications:

1| Generation of phospholipid vesicle-nanotube networks and transport of molecules therein

Jesorka, A ; Stepanyants, N ; Zhang, HJ ; Ortmen, B ; Hakonen, B ; Orwar, O NATURE PROTOCOLS ; 6 (6), 791 - 805 , 2011.

2| Repair of large area pores in supported double bilayers

Gözen, I.; Ortmen, B.; Põldsalu, I.; Dommersnes, P.; Orwar, O.; Jesorka, A. SOFT MATTER; ,9, 2787-2792, JUN 2013

3| Thermal migration of molecular lipid films as a contactless fabrication strategy for lipid nanotube networks

Gözen, I.; Shaali, M.; Ainla, A.; Örtmen, B.; Põldsalu, I.; Kustanovich, K.; Jeffries, G.; Konkoli, Z.; Dommersnes, P.; Jesorka, A. LAB CHIP, 13, 3822-3826, JAN 2013