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Research Article

A Computer Aided Design of an Antistroke Agent Targeting Nitric Oxide Synthase

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ABSTRACT

Stroke is among the leading causes of disability and death. The current treatment involves dissolving clots that occlude arteries that supply the brain. This approach is not only ineffective for strokes due to cerebral hemorrhage but it also predisposes patients into a risk of hemorrhage. This project aimed at contributing efforts towards obtaining new alternatives for combating stroke. The focus was to find an agent that blocks nitric oxide synthase therefore preventing neuronal damage due to hypoxia. Using computer-aided drug design technology, a compound which mimics tetrahydrobiopterin, a natural substrate of niric oxide synthase (NOS), was developed. Computer-based chemical libraries asinex, zick and Parkinson were screened using ligandscout software and 46 ligands, which scored 45 and above, were docked against the binding site of tetrahydrobiopterin in nitric oxide synthase using arguslab on a Pavilion dv4-2160US Entertainment Notebook. Through docking, 4-({[2-(4'-methylbiphenyl-4-yl)ethyl]amino}methyl)-2-methoxy-6-(3H-pyrrol-3-ylmethyl)phenol emerged as a compound which bind best to NOS . Along with this compound, 8 other compounds had pose energies below –11 kcal/mol making them potentially useful in treating stroke. However, nothing was done in this project to check their absorption, distribution, metabolism and toxicological (ADMET) profile. Further studies are therefore recommended on these compounds so as to expound on their potential as antistroke agents

Keywords: Stroke, hemorrhage, nitric oxide synthase (NOS), Arguslab, ADMET

INTRODUCTION

Stroke

Stroke is a disease that affects the blood vessels that supply blood to the brain. A stroke occurs when a blood vessel that brings oxygen and nutrients to the brain either bursts (hemorrhagic stroke) or is clogged by a blood clot or some other mass (ischemic stroke). When a rupture or blockage occurs, parts of the brain don't get the blood and oxygen they need. Without oxygen, nerve cells in the affected area of the brain can't work properly, and die within minutes. When nerve cells can't work, the part of the body they control can't work either. The devastating effects of a severe stroke are often permanent because dead brain cells aren't replaced (Sims and Muyderman, 2009). There are two main types of stroke. The first one is what is referred to as "ischemic stroke" which is caused by blockage of a blood vessel while the other one is called "hemorrhagic stroke" which is caused by bleeding.

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Ischemic stroke

Ischemic stroke is the most common type. It accounts for about 83 percent of all strokes (AHA, 2003). It occurs when a blood clot (thrombus) forms and blocks blood flow in an artery bringing blood to part of the brain. Blood clots usually form in arteries damaged by fatty buildups, called atherosclerosis (Herderscheêet al., 1992). When the blood clot forms within an artery of the brain, it's called a thrombotic stroke. A wandering clot (an embolus) or any other particle that happens to form away from the brain, usually in the heart, may also cause an ischemic stroke. In this case, the stroke is said to result from what is referred to as cerebral embolism. The clot is carried by the bloodstream until it lodges in an artery leading to or in the brain, blocking the flow of blood. The most common cause of these emboli is blood clots that form during atrial fibrillation which is responsible for 15–20 percent of all strokes (Donanet al., 2008). In atrial fibrillation, the atrium quivers instead of beating strongly and effectively. Some blood isn't pumped completely out of them when the heart beats, so it pools and clots can form. When a blood clot enters the circulation and lodges in a narrowed artery of the brain, a stroke occurs. This is called a cardioembolic stroke, i.e. a stroke that occurs because of a heart problem.

Hemorrhagic stroke

A subarachnoid hemorrhage occurs when a blood vessel on the brain's surface ruptures and bleeds into the space between the brain and the skull (but not into the brain itself). A cerebral hemorrhage occurs when a defective artery in the brain bursts, flooding the surrounding tissue with blood (Wang, 2010). Hemorrhage (or bleeding) from an artery in the brain can be caused by a head injury or a burst aneurysm. Aneurysms are blood-filled pouches that balloon out from weak spots in the artery wall .They're often caused or made worse by high blood pressure. Aneurysms aren't always dangerous, but once they burst in the brain, they cause a hemorrhagic stroke. When a cerebral or subarachnoid hemorrhage occurs, the loss of a constant blood supply means some brain cells no longer can work. Accumulated blood from the burst artery also may put pressure on surrounding brain tissue and interfere with how the brain works Severe or mild symptoms can result, depending on the amount of pressure.

Molecular pathophysiology of stroke

Both types of stroke are featured by a decreased supply of blood and thus oxygen and other nutrients to brain cells leading to cellular death due to ischemia. This causes a depletion of energy stores which is the primary pathologic mechanism in stroke. In ischemic stroke, the blood supply to the brain is disrupted by cerebrovascular disease while in hemorrhagic stroke rupture of arteries is the cause of decreased blood flow. The final event in cerebral ischemia is the death of neurons, resulting in irreversible loss of neurologic function (Beauchamp and Bryan, 1998). Studies based on animal and tissue culture models of ischemia have demonstrated that occurrence of many secondary biochemical changes that exacerbate injury, occur in response to the initial insult (Lo et al., 2005). In models of cerebral ischemia in rodents, as much as 50% or more of ischemic brain may be spared from infarction by preventing these secondary biochemical events (Warner et al., 2004). Understanding of the mechanisms by which neuronal cell death takes place has resulted in a number of therapeutic strategies that aim to prevent secondary biochemical changes and thus decrease the damage that results from cerebral ischemia. Ischemic neuronal death may involve the activation of enzymes and receptors that are constitutively expressed in brain. These existing receptors and enzymes do not require energy or the synthesis of new protein to exacerbate necrotic cell death. These mechanisms are appealing targets for therapeutic intervention because they may occur hours or days after the initiation of ischemia.

Mechanisms of necrotic cell death

Besides energy depletion that is common in stroke, Excitatory Amino Acids (EAAs) like glutamate intensify ischemic injury (Bano et al., 2005; Castillo et al., 1996). After cerebral ischemia, the level of excitatory amino acids increase significantly and cause an increase in intraneuronal cytosolic Ca^{2+} concentrations by opening the N methyl-D-aspartate (NMDA) channels. These channels are responsible for calcium ion entry into the neurons an event which is immediately lethal (Beal, 1992). The toxic effects of increased intracellular calcium are mediated by calcium-dependent or calcium-

induced enzymes like nitric oxide synthase, cycloxygenase, phospholipase A2, and calpain 1 (Yamashima et al., 2004). Calpain 1 is a calcium-activated protease which is linked to glutamate receptors in hippocampus and participates in the conversion of xanthine dehydrogenase to xanthine oxidase, which metabolizes xanthine to its reactive oxygen species, superoxide.

When phospholipase A2 is activated by calcium, arachidonic acid is released from injured cell membranes. This acid is then metabolized by the enzyme cyclooxygenase into a prostaglandin, PGH2. During the process superoxide ions are formed as by-products of arachidonic acid metabolism. Intracellular calcium can also activate calcium-dependent isoforms of nitric oxide synthase to produce nitric oxide which combines with the superoxide produced as the byproduct of cyclooxygenase, xanthine oxidase, or other sources to form the highly reactive species peroxynitrite, which exacerbates tissue damage (Eliasson,1999). Therefore, EAA-mediated elevation of intracellular calcium concentrations activates both cyclooxygenase and nitric oxide synthase, which then synergistically contribute to ischemic brain injury (Nakka et al., 2008).

Literature Review

Effects and epidemiology of stroke

A stroke refers to a quick loss of brain functions due to a disruption in the blood supply to the brain. This disruption of blood flow can be due to a hemorrhage (leakage of blood) or ischemia (lack of blood flow due to a blockage). Strokes, or brain attacks, are a major cause of death and permanent disability (Goodman et al., 2003). They occur when blood flow to a region of the brain is obstructed and may result in death of brain tissue. Some of the most common disabilities that result from a stroke are speech and memory loss, cognitive deficits and paralysis on one side of the body. Many times this paralysis results in hypertonicity (or high levels of stiffness and high muscle tone) on the affected side. According to Matherset and co-workers (2009), stroke was the second most common cause of death worldwide in 2004, resulting in 5.7 million deaths (~10% of the total). Approximately 9 million people had a stroke in 2008 and 30 million people have previously had a stroke and are still alive (Matherset et al., 2009). Stroke is the third leading cause of death and the leading cause of disability in the United States. Approximately 600,000 strokes, or brain attacks, occur in the United States each year and of these, approximately 150,000 (25 percent) are fatal. The incidence of stroke is higher in Afro-Americans than Caucasians (American Heart Association, 2003; Stanley, 2013). Stroke occurs at an equal rate in men and women, but women are more likely to die from stroke. Ischemic stroke occurs more frequently in people over age 65 while hemorrhagic stroke is more common in younger people (Stanley, 2013).

Current treatment of stroke

Medications used to treat stroke include thrombolytics, antiplatelets and anticoagulants. Also used are drugs that lower pressure and cholesterol to minimize the chance of atherosclerosis (NINDS, 2009).

Thrombolytics

These are used to treat acute ischemic stroke where the immediate goal is to break apart the offending clot, a process known as thrombolysis. The main drug used for this purpose is tissue plasminogen activator (tPA).

Antiplatelets

These are given immediately after the onset of a stroke because they decrease the chances of another stroke. They do so by preventing blood clot formations in blood vessels. When a blood clot forms, it can lodge in brain's blood vessels, deprive it of oxygen and result in a serious and sudden stroke.

Anticoagulants

These may be given orally or intravenously. These drugs work by thinning the blood and preventing clotting. They are also used for treatment and prevention of deep vein thromboses and pulmonary emboli. Anticoagulants can help prevent a variety of potentially life-threatening conditions for which

individuals with stroke are at risk, such as myocardial infarction, pulmonary embolism, and deep vein thrombosis-which are caused by clots in the heart, lungs and deep veins of the legs, respectively (Jenny, 2012).

Shortcomings of the current treatment

Thrombolytics

Treatment with tPA interferes with blood clotting and has also been shown to increase leaking along the blood-brain barrier thus carrying the risk of intracerebral hemorrhage. Therefore, tPA not recommended for some people, such as those with a history of brain hemorrhage or significantly elevated blood pressure (greater than 185/110 mmHg). The risk of tPA-induced hemorrhage increases over time from stroke onset, which has limited its use to the first three hours after stroke (Armenian medical network, 2005). Another problem facing tPA therapy is that less than 5% of patients who might benefit from this drug reaches in time to receive treatment. According to the American Stroke Association, this is due to lack of knowledge about stroke symptoms and necessity of urgent treatment. This fact was also mentioned by Jeanie who explained that only about 2% of stroke patients receive tPA because it must be given within three hours of symptom onset (Jeanie, 2002).

Antiplatelets and anticoagulants

Warfarin is a routine medication for preventing strokes. But it is inconvenient for some patients because careful monitoring and regular blood tests are needed to prevent excessive bleeding from cuts or stomach ulcers for which frequent clinic visits are required (NICE, 2012). Either, use of aspirin is contraindicated in some patients e.g. those with active peptic ulcers.

Thus, it can be concluded that all the current medications for stroke, carry a risk of hemorrhage. Use of warfarin causes inconvenience due to a requirement for close monitoring. Furthermore intra-arterial administration of tPA need special expertise and equipment, which are lacking in most hospital settings. It is in light of these facts that a need for alternative drugs for stroke arises.

Nitric oxide synthase as a potential target for treating stroke

Hypoxic condition that follows ischemia, whether due to ischemic and hemorrhagic stroke, triggers a cascade of events that end up with destruction of brain cells. Nitric oxide synthase which catalyses the formation of nitric oxide is among important actors in this pathological chain. According to Cacha and his colleagues, excess nitric oxide produced in hypoxia rapidly reacts with superoxide free radicals to form peroxynitrite free radicals which facilitates the nitration of lipids, DNA and proteins culminating into damaging the neurons (Cacha et al., 2002). Chacha and co-workers (2002) showed that inhibition of nitric oxide synthase by 2-imino biotin had a protective effects on neurons. A previous study about the role of nitric oxide synthase in chronic glaucoma by Arthur and co-workers (1999) portrayed similar results. The authors pointed out that excessive nitric oxide produced by astrocytes led to death and loss of retinal ganglion cell axons. These researchers went further to demonstrate that inhibition of inducible nitric oxide synthase by aminoguanidine prevents degeneration of axons in optic disc (Arthur et al., 1999).

Commenting on their findings, Paul (1999) explained that "beyond glaucoma, inhibition of nitric oxide synthase will prove helpful in other neurovascular diseases". The above literature shows that inhibiting nitric oxide synthase provides neuroprotection which is mentioned by various authors as a basis for a search of new medicines against stroke (Love, 1999; Warner et al., 2004). This project targeted the binding site of tetrahydrobiopterin as a basis for developing a new agents that inhibit NOS and thus providing protection against stroke .

Efforts to design drugs that inhibit nitric oxide synthase

Prolonged NO production by overstimulation of nitric oxide synthase has been proved to cause diseases like stroke and septic shock (Ulrich et al ,. 2011). Several classes of potent NOS inhibitors have been reported, most of them targeting the arginine binding site of the enzyme.
An example of such drugs is N^5-(1-imino-3-butenyl)-L-ornithine (Boga and Owen,1998).

Other drugs designed to target the enzyme are as shown in Figure 1.

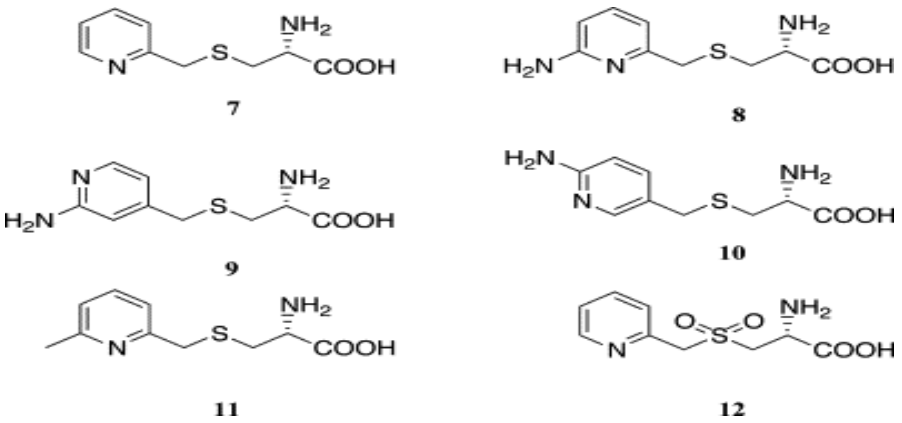


Figure 1: Nitric oxide synthase inhibitors based on L-amino acids containing pyridine moiety.

These drugs, all of which are amino acids, were designed and synthesized by Ryosuke and his colleagues in 2006. Based on their 2006 report, 2-aminopyridine-containing l-amino acids (**8)** had potent inhibitory activity toward all of the human NOS isozymes (Ryosuke et al., 2006). In another study, Aparna and co-workers (2007) carried out molecular dynamics (MD) simulations for inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) isoforms complexed with substrate (l-arginine) and the iNOS specific inhibitor GW 273629 (**2)**. Thereafter ,they used the results to synthesize compound 3 and 4 as shown in Figure 2.

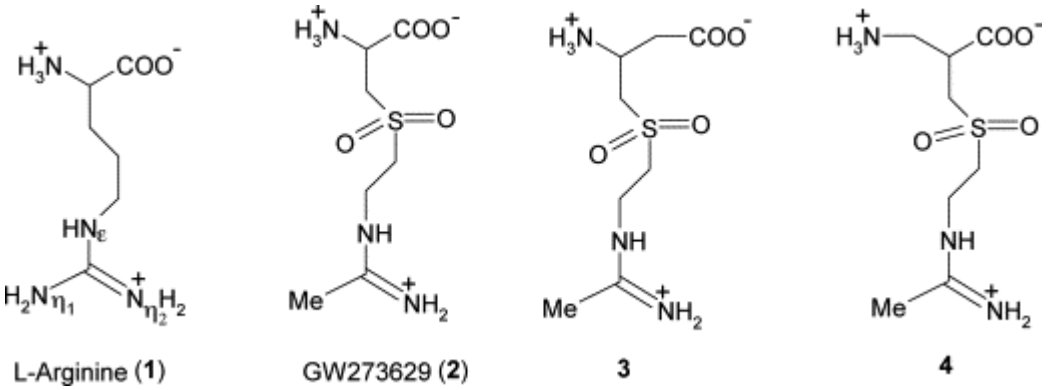


Figure 2: Arginine-based NOS inhibitors.

Analysis of the binding patterns of these compounds showed molecule **4** to be more selective to iNOS when compared to eNOS (Aparna et al., 2007).

Figure 3 shows the drugs that were designed by Ulrich and co-workers (2011) as potential iNOS inhibitors of the inducible form of nitric oxide synthase. These compounds were developed based on L-arginine.

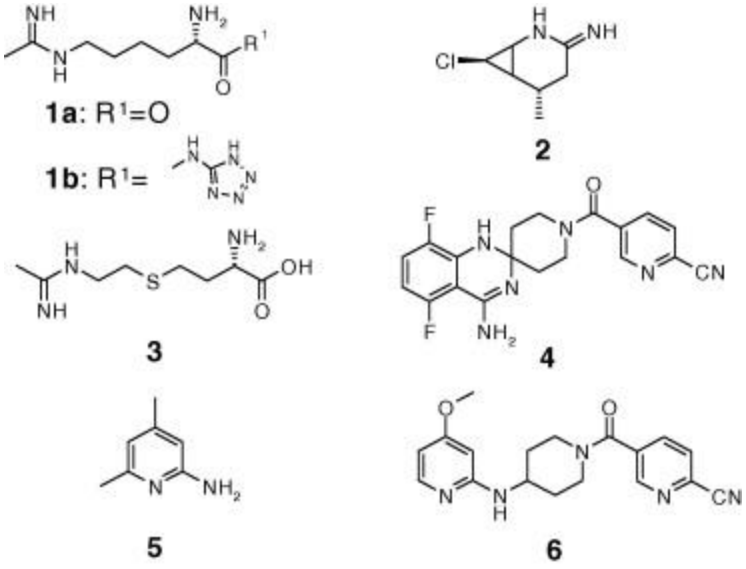


Figure 3: L-arginine-based NOS inhibitors.

Non-amino acid inhibitors of nitric acid synthase that have been developed include those based on a cis-pyrrolidinepharmacophore (Figure 4). However, despite being selective inhibitors of neural NOS, subsequent analysis of these compounds revealed that they were not only poor in CNS penetration but also they had low potency (Fengtianet al., 2010).

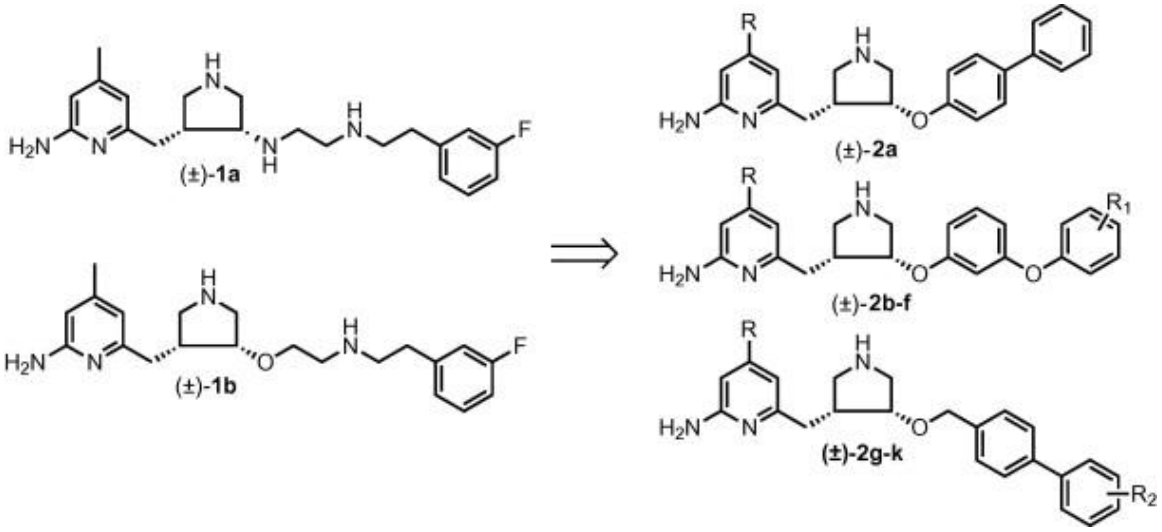


Figure 4: Cis-pyrrolidine-based NOS inhibitors.

In 2012, Ghadeer and co-workers carried out a virtual screening of NCI database. In this study, 143 iNOS inhibitors were collected and their pharmacophoric space explored. QSAR analysis was employed to select optimal pharmacophore models and then the database was screened for new iNOS inhibitors using pharmacophore models and associated QSAR model. Three hits were identified which exhibited nanomolar and low micromolar IC_{50} values (Ghadeer et al., 2012). The most potent hit exhibited irreversible inhibition of iNOS with IC_{50} value of 1.4 nM .Their structures are shown in the Figure 5. Structure C is the one with IC_{50} value of 1.4 nM

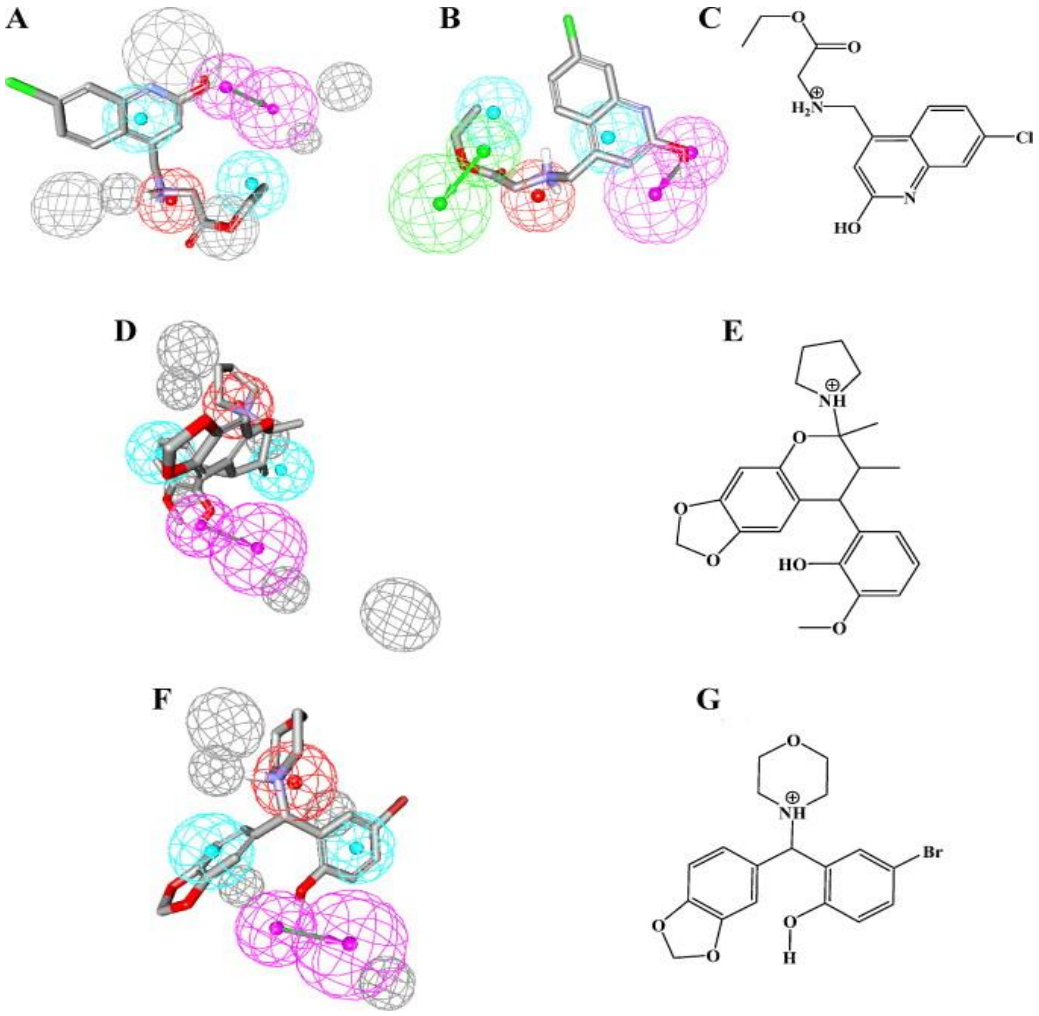


Figure 5: Hits with nanomolar and low micromolar IC 50 values.

Marc and co-workers (2007) designed pyridine-based inhibitors of nitric oxide formation. The unsubstituted pyridine (**2a**) had an IC_{50} of 78 nM (Table 1) while substitution as in **2b** led to a significant reduction in potency. In the same study, pyrimidines were also investigated as central cores. Results showed that compounds in which one of the key substituent is straddled by nitrogen (**2c**–**e**), had almost the same potency as unsubstituted pyridine. The single 4,6-disubstituted compound, **2f**, was inactive. Unlike the pyridine, additional substitution of the pyrimidine gave a compound with similar activity (**2d**).

Table 1. Inhibition of NO production in A172 cells

Compound	X	Y	Z	R	A172 (nM) IC_{50}
2a	N	CH	CH	H	78
2b	N	CF	CF	CH_3	450
2c	N	CH	N	H	55
2d	N	CH	N	CH_3	90
2e	N	N	CH	H	74
2f	CH	N	N	H	>10,000
2g	N	CCH_3	N	H	130

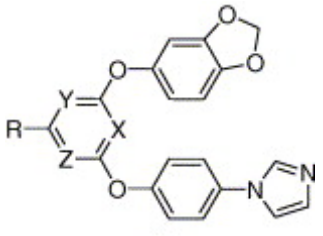
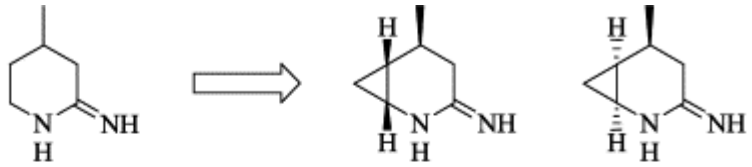


Figure 6: Pyridine based NOS inhibitors.

Another class of compounds that has shown good activity against NOS is that of 2-iminopiperidines fused to small-membered rings (Yasufumiet al., 2003). The potency of bicyclic compounds **5**–**9** (Table 2) was virtually the same as that of compound **1** in the hiNOS inhibition assay. However, the 1-methyl analogues **8** and **9** showed better isoform selectivity than their corresponding unsubstituted analogues **7** and **6**, respectively.

Table 2a. IC 50 and heNOS/hi NOS selectivity



	1	**6**	**7**
iNOS IC_{50} (μM)	0.14	0.087	0.047
Selectivity heNOS/hiNOS	7.9	2.1	3.6

Table 2b. IC 50 and heNOS/hi NOS selectivity

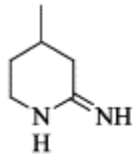
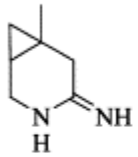
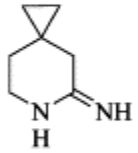
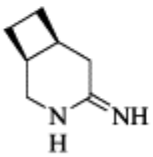
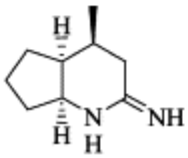
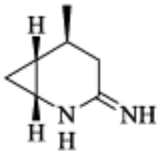
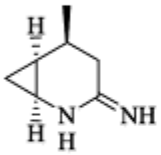
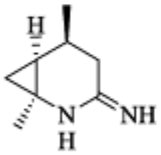
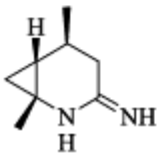
Structure	Compound	hiNOS IC_{50} (μM)	heNOS IC_{50} (μM)	Selectivity heNOS/hiNOS
	**1** [b]	0.14	1.1	7.9
	**2**	2.2	2.0	0.9
	**3**	0.20	0.34	1.7
	**4**	0.74	4.4	5.9
	**5** [c]	0.10	0.76	7.6

Table 2c. IC 50 and heNOS/hi NOS selectivity

Structure	Compound	hiNOS IC_{50} (μM)	heNOS IC_{50} (μM)	Selectivity heNOS/hiNOS
	**6** (*anti*)	0.087	0.18	2.1
	**7** (*syn*)	0.047	0.17	3.6
	**8** (*syn*)	0.22	1.60	7.3
	**9** (*anti*)	0.13	0.61	4.7

The same authors also reported discovery of α,β-unsaturated cyclic amidines, as selective inhibitors of inducible nitric oxide synthase (iNOS). After their synthesis, dihydropyridin-2(1H)-imines and 1,5,6,7-tetrahydro-2H-azepin-2-imines were evaluated using a nitric oxide synthase inhibition assay (Yasufumiet al., 2003). After analysis, the dihydropyridin-2(1H)-imines **1**, **9**–**11** and the 1,5,6,7-tetrahydro-2H-azepin-2-imines **14**, **16** (Figure 7) were identified as potent inhibitors of inducible nitric oxide synthase.

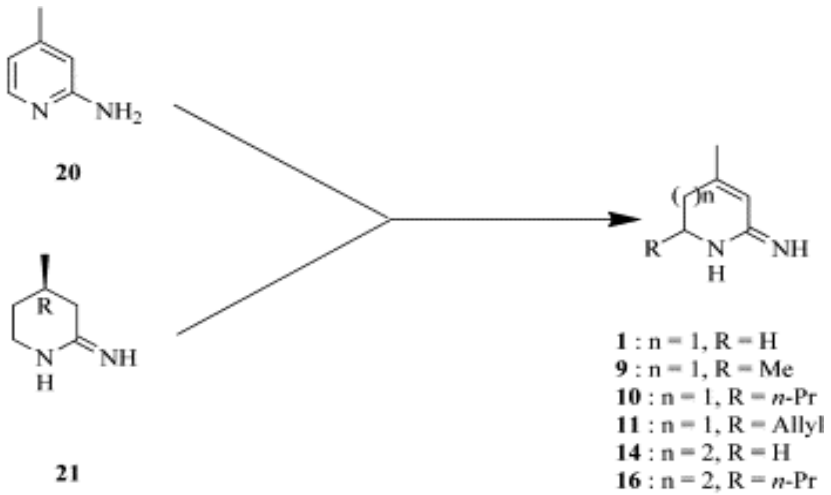


Figure 7: NOS inhibitors based on α,β-unsaturated cyclic amidines.

Several 5-phenyl-1H-pyrrole-2-carboxamide derivatives have also been synthesized by Luisa and co-workers (2009) and their potential to inhibit both neural and inducible Nitric Oxide Synthase (nNOS and iNOS) evaluated. Results identified 5-(2-aminophenyl)-1H-pyrrole-2-carboxilic acid methylamide and cyclopentylamide as potent compounds. Observed in the MPTP model of Parkinson's disease is that both compounds prevented the increment of the inducible NOS activity in both cytosol (iNOS) and mitochondria (i-mtNOS).

Figure 8: NOS inhibitors based on 5-phenyl-1H-pyrrole-2-carboxamide.

From the above literature, it is clear that much of the efforts to develop NOS inhibitors have concentrated in designing compounds that target the binding site of L- arginine. This project therefore aimed at widening the chances of finding new inhibitors of nitric oxide synthase by focusing on the binding site of tetrahydrobiopterin. Tetrahyrobiopterin is among the natural ligands of NOS. Inhibition of its binding site has a potential to provide neuroprotection which is useful in management of stroke. Developed in this project are compounds that block NOS- catalysed production of NO by inhibiting binding site of tetrahydrobiopterin.

METHODOLOGY

Target Identification

The first step in modern drug discovery is usually identification of a disease target which is in most cases a protein involved in the disease process. This project used nitric oxide synthase as its disease target.

Lead Identification

In this project lead compound was determined using a ligand-based technique. Ligand-similarity based lead identification is a technique which follows the principle of similarity. The technique operates under the assumption that molecules with a similar structure will have similar chemical properties. That means the information provided by a compound or set of compounds known to bind to the desired target can be used to identify new compounds from the external databases of chemical compounds with the aid of virtual screening approaches. Natural ligand used in this project was tetrahydrobiopterin. Chemical libraries asinex, zinc and Parkinson were screened using ligandscout software and compounds that scored 45 and above were docked against the receptor (NOS) using arguslab on a Pavilion dv4-2160US Entertainment Notebook.

Docking

The following steps in molecular docking were followed;

Step I – Building the receptor
In this step the 3D structure of the receptor was downloaded from PDB and modified by removal of the water molecules from the cavity.

Step II – Identification of the active site
After the receptor was built, the active site within the receptor was identified. The enzyme nitric oxide synthase has many active sites but the one for tetrahydrobiopterin was selected.

Step III – Ligand preparation
Ligands obtained from various databases like zinc were sketched using arguslab on a Pavilion dv4-2160US Entertainment Notebook.

Step IV- Docking
Finally, the ligands were docked onto the receptor and their pose energies recorded.

Data Collecting Methods/Tools

Methods that were used in data collection include virtual screening using ligandscout and docking by using arguslab on a Pavilion dv4-2160US Entertainment Notebook. While virtual screening was used to get information about compounds that are similar to the natural ligand, docking helped to collect data about best pose energies of docked molecules. Pose energy is related to the binding strength of the ligand to the receptor. The higher the negative value the higher the stability of the ligand-receptor complex that is formed.
Tools that were used at different stages of the project and the purpose of each tool are shown in Table 3.

Table 3. Tools used in the project

SN	Tool / database to be used	Use
1	PDB	Source of Protein (nitric oxide synthase) structure
2	Zinc	Ligand library
3	Ligandscout	Hits and lead identification
4	Arguslab	Docking
5	Chemipack	Drawing chemical structures

RESULTS

This project had four objectives namely; natural ligand identification, virtual screening, docking of compounds obtained through screening of chemical libraries and docking of NOS inhibitors reported in literature. Results obtained are presented based on the objectives of project under the following subheadings.

Natural Ligand Identification
The natural ligand of the enzyme nitric oxide synthase was identified by going through different literature and its structure as is shown in Figure 9.

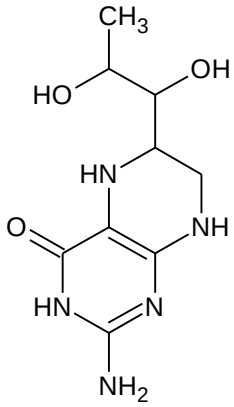
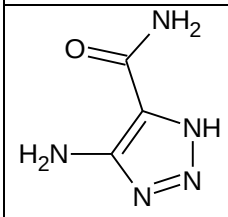
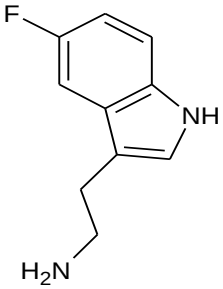
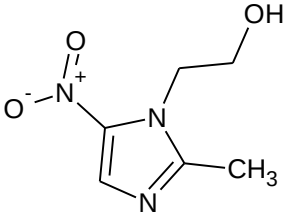


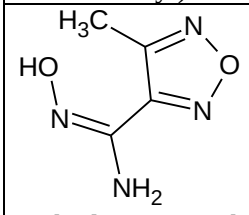
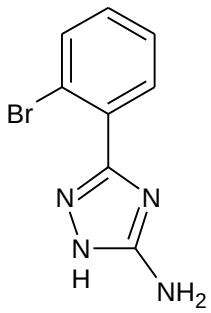
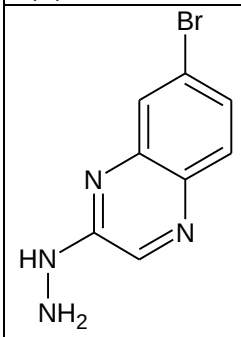
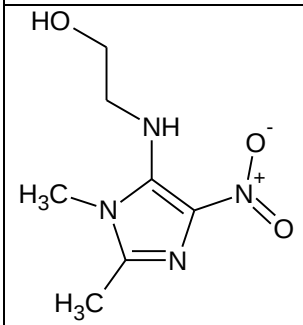
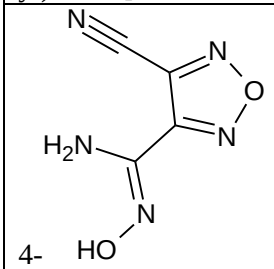
Figure 9: Tetrahydrobiopterin

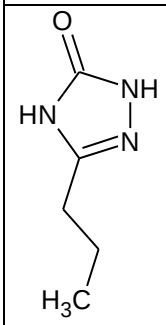
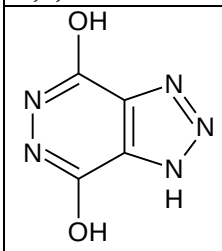
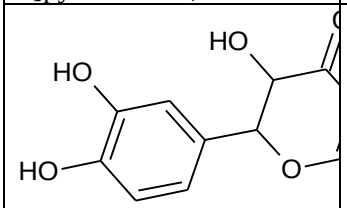
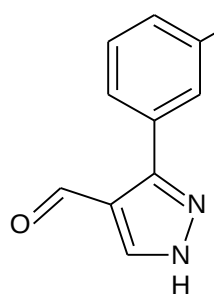
Virtual Screening

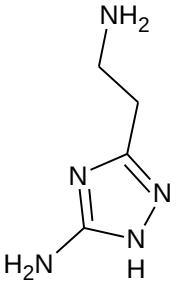
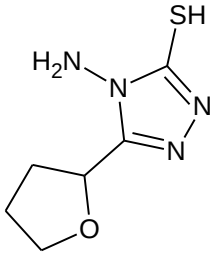
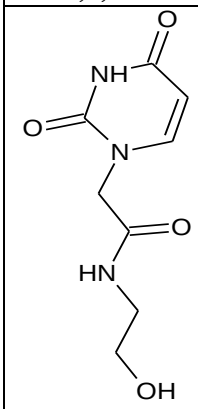
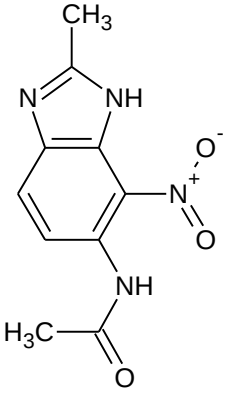
The purpose of virtual screening was to obtain compounds with high similarity to tetrahydrobiopterin, a natural ligand of nitric oxide synthase which was the target enzyme of the project. Three chemical libraries were screened using ligandscout software and compounds scoring 45 and above recorded. Structures of such compounds with their corresponding scores are depicted in Table 4.

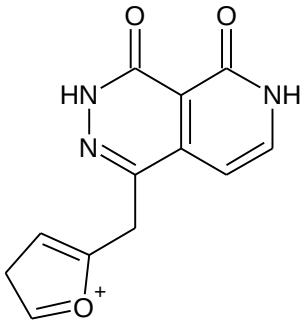
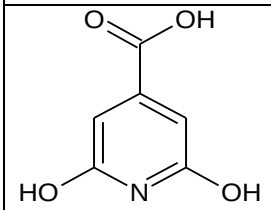
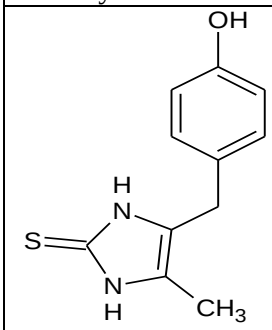
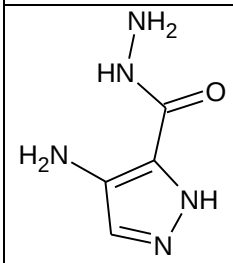
Table 4. Results of the screening process

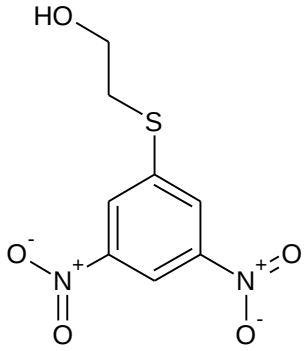
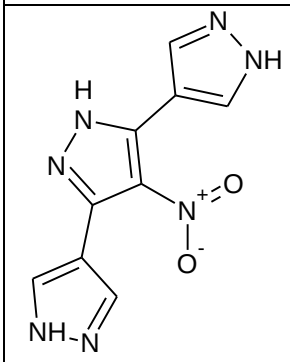
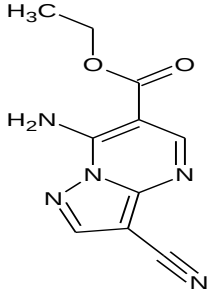
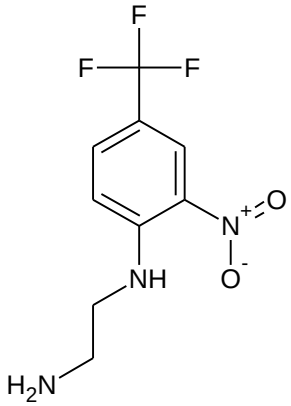
Chemical Name	Ligands cout score	Hydrogen bond acceptor	Hydrogen bond donor	Hydrogen bond acceptor/ donor	No. of hydrophobic atoms	No. of polar atoms
 4-amino-1H-1,2,3-triazole-5-carboxamide	76.35	2		2	1	4
 2-(5-fluoro-1H-indol-3-yl)ethanamine	67.27	-	1	1	7	4
 2-(2-methyl-5-nitro-1H-	75.55	4	-	1	1	5

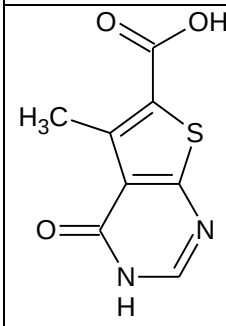
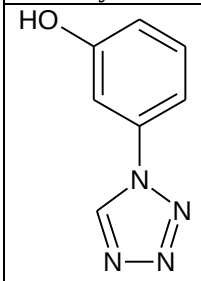
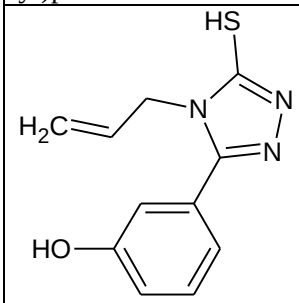
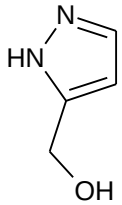
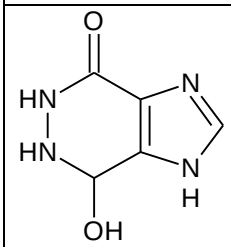
imidazol-1-yl)ethanol						
 N'-hydroxy-4-methyl-1,2,5-oxadiazole-3-carboximidamide	65.82	3	-	2	-	5
 3-(2-bromophenyl)-1H-1,2,4-triazol-5-amine	65.95	2	-	2	6	2
 7-bromo-2-hydrazinylquinoxaline	65.77	2	-	2	5	4
 2-[(1,2-dimethyl-4-nitro-1H-imidazol-5-yl)amino]ethanol	56.65	4	-	2	-	7
 4-	65.87	3	-	2	-	5

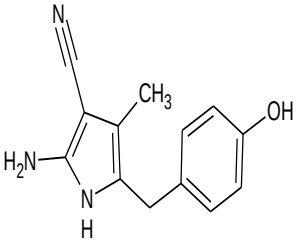
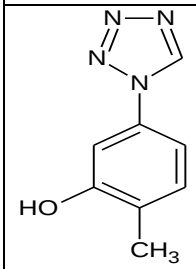
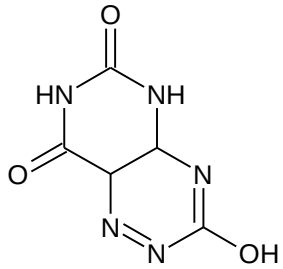
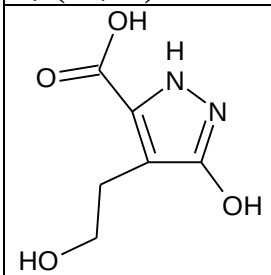
4-cyano-N'-hydroxy-1,2,5-oxadiazole-3-carboximidamide						
 5-propyl-2,4-dihydro-3H-1,2,4-triazol-3-one	57.27	1	-	3	-	5
 1H-[1,2,3]triazolo[4,5-d]pyridazine-4,7-diol	76.71	-	-	3	-	8
 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-2,3,5,6-tetrahydro-4H-chromen-4-one	75.24	1	-	5	7	9
 3-(3-hydroxyphenyl)-1H-pyrazole-4-carbaldehyde	66.70	1	-	2	6	5

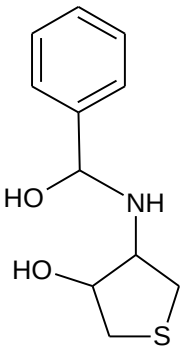
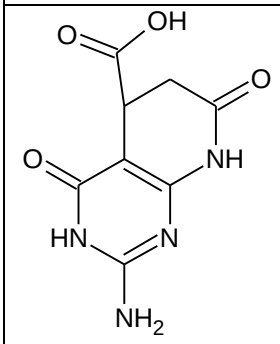
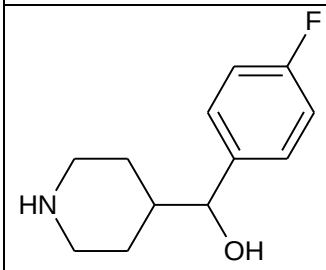
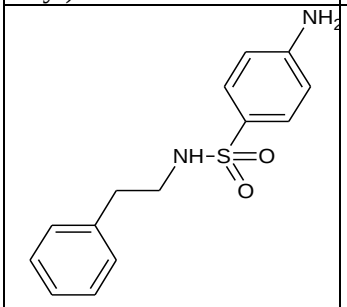
 3-(2-aminoethyl)-1H-1,2,4-triazol-5-amine	63.82	1	-	3	1	3
 4-amino-5-(tetrahydrofuran-2-yl)-4H-1,2,4-triazole-3-thiol	56.23	3	-	1	2	5
 2-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-N-(2-hydroxyethyl)acetamide	76	1	-	3	1	10
 N-(2-methyl-7-nitro-1H-benzimidazol-6-yl)acetamide	66.92	1	-	3	4	9

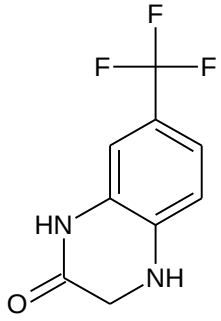
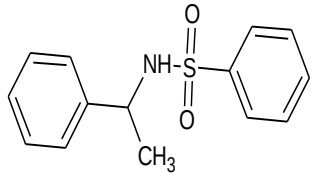
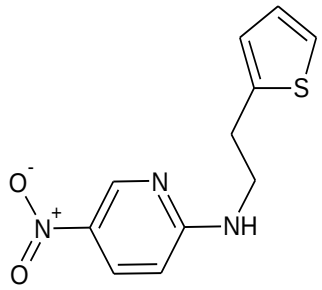
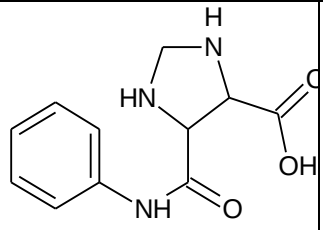
 5-[(4,5-dioxo-3,4,5,6-tetrahydropyrido[3,4-d]pyridazin-1-yl)methyl]-3H-furanium	56	1	-	2	5	9
 2,6-dihydroxypyridine-4-carboxylic acid	57.73	1	-	3	3	4
 4-(4-hydroxybenzyl)-5-methyl-1,3-dihydro-2H-imidazole-2-thione	77.16	1	-	3	7	4
 4-amino-1H-pyrazole-5-carbohydrazide	58.2	-	-	5	-	5

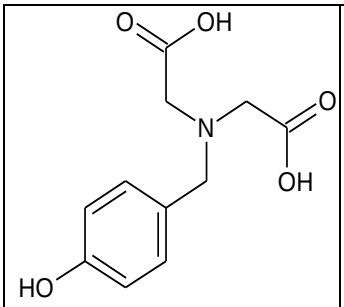
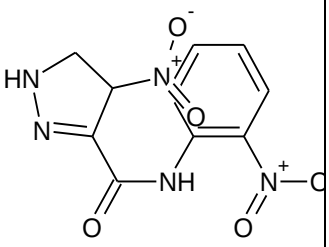
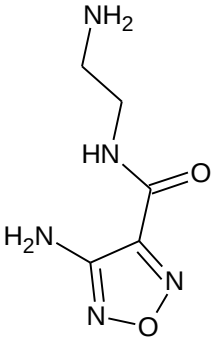
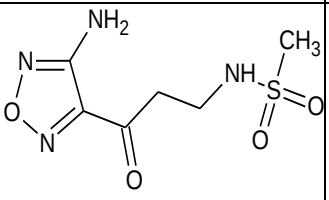
 2-[(3,5-dinitrophenyl)sulfanyl]ethanol	63	2	-	1	6	7
 4'-nitro-1H,1'H,1''H-4,3':5',4''-terpyrazole	76.31	5	-	3	2	8
 ethyl 7-amino-3-cyanopyrazolo[1,5-a]pyrimidine-6-carboxylate	65.84	2	-	2	3	8
 N-[2-nitro-4-(trifluoromethyl)phenyl]ethane-1,2-diamine	65.70	1	-	2	5	9

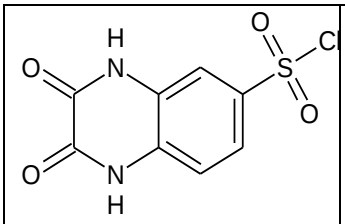
 5-methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidine-6-carboxylic acid	66.09	1	-	2	5	6
 3-(1H-tetrazol-1-yl)phenol	75.75	2	-	1	4	3
 3-[4-(prop-2-en-1-yl)-5-sulfanyl-4H-1,2,4-triazol-3-yl]phenol	75.95	2	-	3	7	4
 1H-pyrazol-5-ylmethanol	75.91	1	-	2	1	3
 7-hydroxy-1,5,6,7-tetrahydro-4H-	76.09	-	-	3	-	8

imidazo[4,5-d]pyridazin-4-one						
 2-amino-5-(4-hydroxybenzyl)-4-methyl-1H-pyrrole-3-carbonitrile	65	-	-	3	9	5
 2-methyl-5-(1H-tetrazol-1-yl)phenol	75.76	4	-	1	5	3
 3-hydroxy-4a,8a-dihydropyrimido[4,5-e][1,2,4]triazine-6,8(5H,7H)-dione	76.82	-	-	4	-	9
 3-hydroxy-4-(2-hydroxyethyl)-1H-pyrazole-5-carboxylic acid	76.39	1	-	4	2	5

 4-{[hydroxy(phenyl)methyl]amino}tetrahydrothiophene-3-ol	76.74	-	-	4	2	5
 2-amino-4,7-dioxo-3,4,5,6,7,8-hexahydropyrido[2,3-d]pyrimidine-5-carboxylic acid	53.21	-	-	5	3	7
 (4-fluorophenyl)(piperidin-4-yl)methanol	45	-	-	2	9	4
 4-amino-N-(2-	46	-	-	1	13	4

phenylethyl)benzenesulfonamide						
 7-(trifluoromethyl)-3,4-dihydroquinoxalin-2(1H)-one	55.88	-	-	2	5	8
 N-(1-phenylethyl)benzenesulfonamide	46	-	-	2	5	12
 5-nitro-N-[2-(thiophen-2-yl)ethyl]pyridin-2-amine	57.60	1	-	1	8	7
 5-(phenylcarbamoyl)imidazolidine-4-carboxylic acid Best pose energy : -10.2 kcal/mol	67.92	1	-	3	5	7

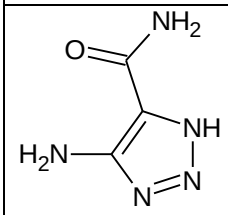
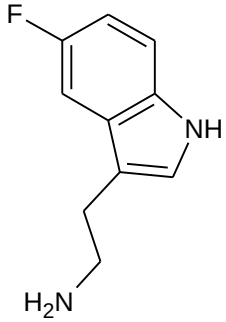
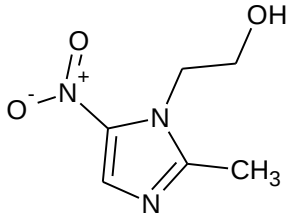
 2,2'-[(4-hydroxybenzyl)imino]diacetic acid	47.02	2	-	4	6	14
 4-nitro-N-(2-nitrophenyl)-4,5-dihydro-1H-pyrazole-3-carboxamide	56.96	6	-	2	4	9
 4-amino-N-(2-aminoethyl)-1,2,5-oxadiazole-3-carboxamide	76.49	3	-	3	-	6
 N-[3-(4-amino-1,2,5-oxadiazol-3-yl)-3-oxopropyl]methanesulfonamide	65.71	3	-	3	1	7

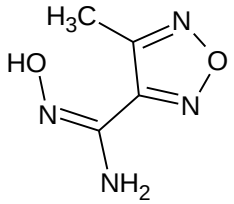
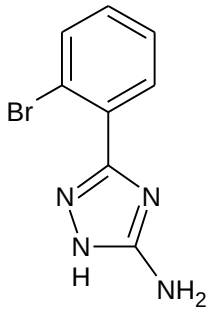
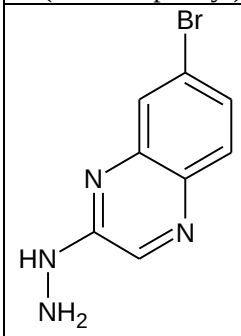
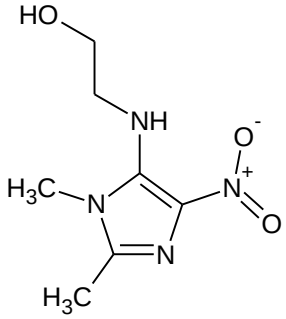
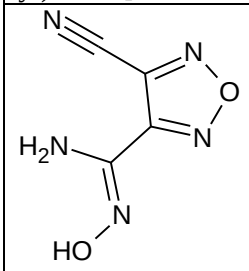
 2,3-dioxo-1,2,3,4-tetrahydroquinoxaline-6-sulfonyl chloride	75.50	-	-	2	5	9

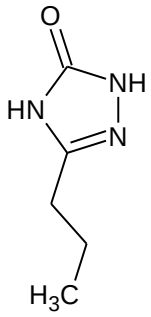
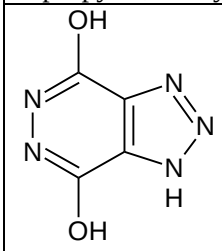
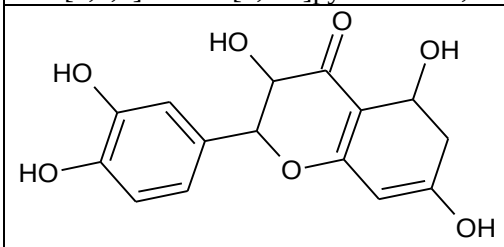
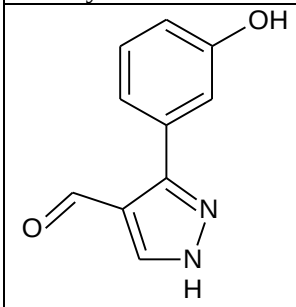
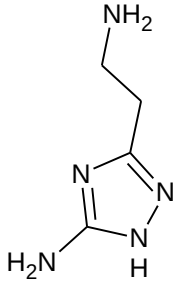
Docking

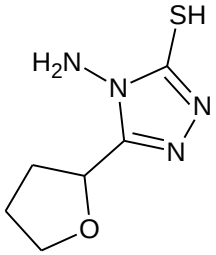
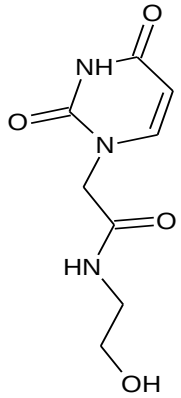
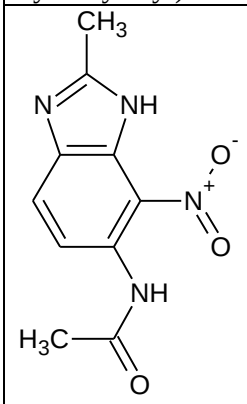
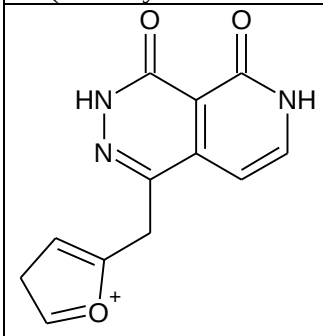
In order to identify compounds with the lowest pose energy, docking was carried out using arguslab on a Pavilion dv4-2160US Entertainment Notebook.. As shown in Table 5, pose energies ranged from -4 to -10.7 kcal/mol. With modifications of the ligands with pose energy -10.5 and -10.7 kcal/mol, pose energy was lowered to below -11 kcal/mol.

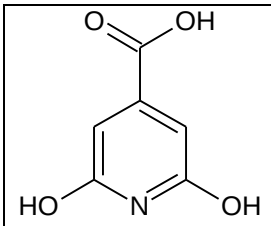
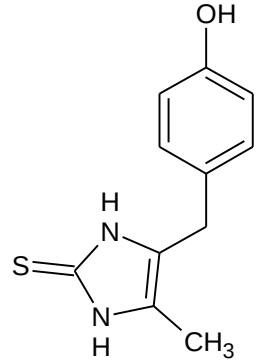
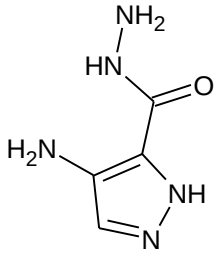
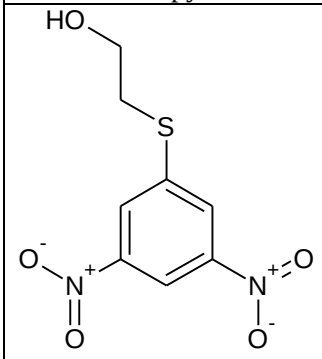
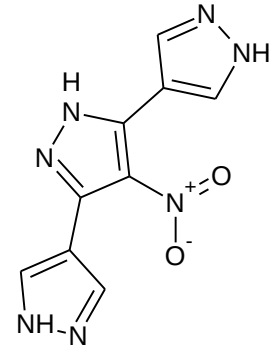
Table 5. Structures of docked compounds and their corresponding pose energies

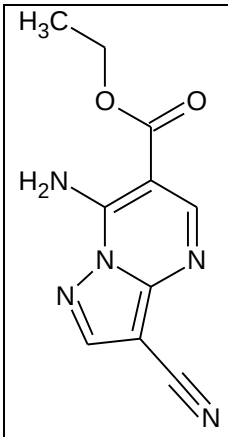
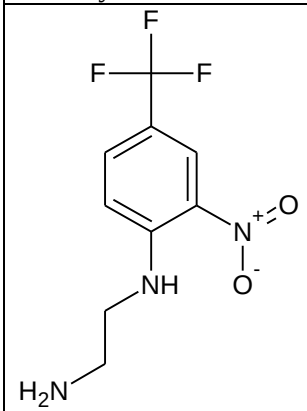
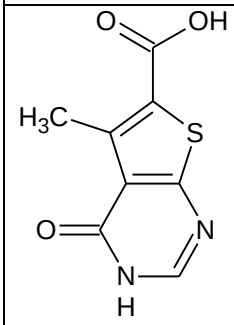
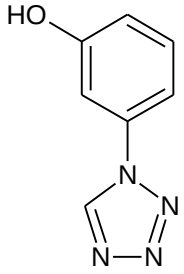
Ligand structure	**Best pose energy (kcal/mol)**	**Residues with which it forms hydrogen bonds**
 4-amino-1H-1,2,3-triazole-5-carboxamide	-4	116leusine, 124 serine and 109 threonine
 2-(5-fluoro-1H-indol-3-yl)ethanamine	-5.5	462 isoleusine and 465 valine
 2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethanol	-6.01	1334 glutamic acid

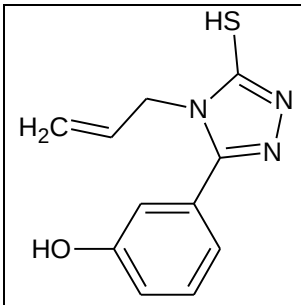
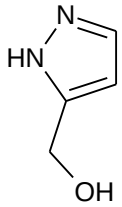
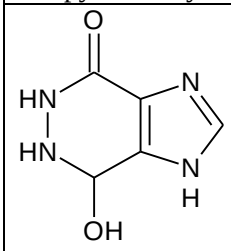
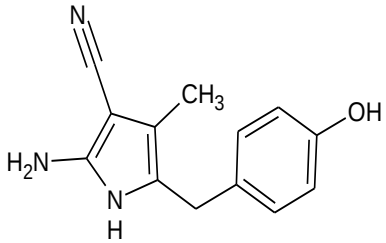
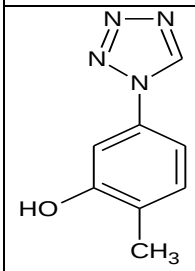
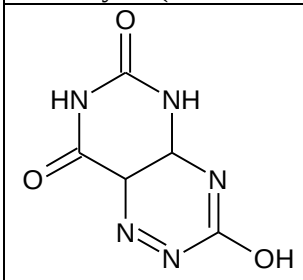
 N'-hydroxy-4-methyl-1,2,5-oxadiazole-3-carboximidamide	-7.3	aspartate and cysteine
 3-(2-bromophenyl)-1H-1,2,4-triazol-5-amine	-9.93	882 isoleusine, 885 valine and 896 proline
 7-bromo-2-hydrazinylquinoxaline	-8.9	467 proline and 466 proline
 2-[(1,2-dimethyl-4-nitro-1H-imidazol-5-yl)amino]ethanol	-6.3	residue 17177 (no name)
 4-cyano-N'-hydroxy-1,2,5-oxadiazole-3-carboximidamide	-7.5	459 alanine and 535 cysteine

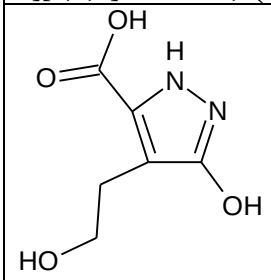
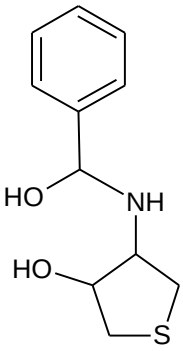
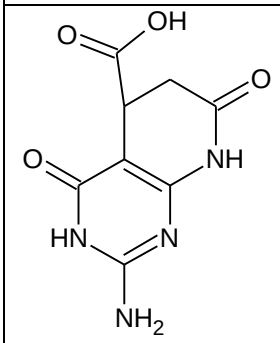
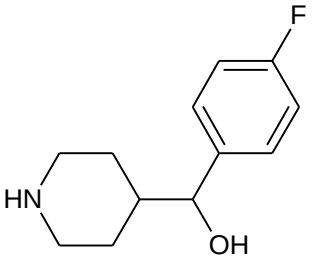
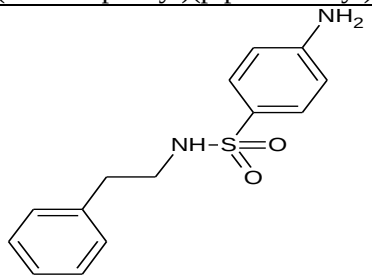
 5-propyl-2,4-dihydro-3H-1,2,4-triazol-3-one	-7.2	1779 HEM and 1222 aspartate
 1H-[1,2,3]triazolo[4,5-d]pyridazine-4,7-diol	-6.77	1194 ASN , 1102 alanine and 1779 HEM,
 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-2,3,5,6-tetrahydro-4H-chromen-4-one	-10.4	1736 phenylalanine, 1302 isoleusine and 1779 HEM
 3-(3-hydroxyphenyl)-1H-pyrazole-4-carbaldehyde	-9.16	1322 alanine
 3-(2-aminoethyl)-1H-1,2,4-triazol-5-amine	-8.0	124 serine, 109 threonine,and 460 aspartate

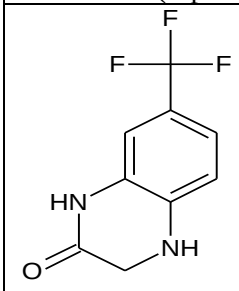
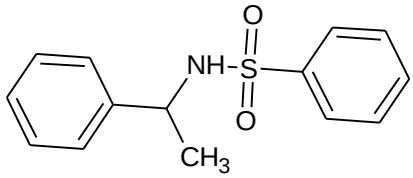
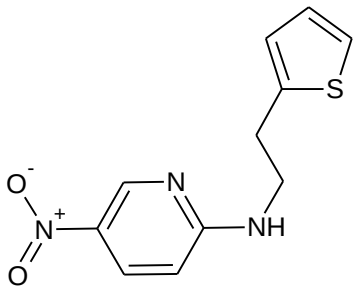
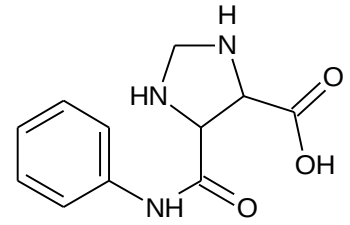
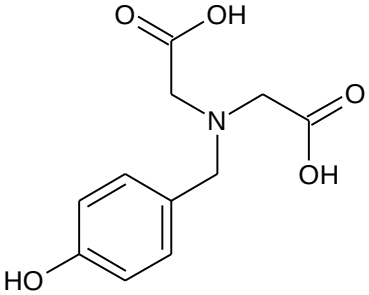
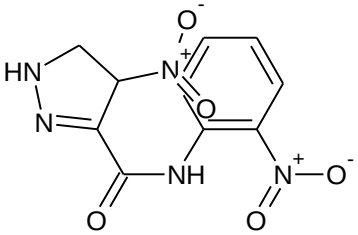
 4-amino-5-(tetrahydrofuran-2-yl)-4H-1,2,4-triazole-3-thiol	-7.2	1375 cysteine,and 955 cysteine
 2-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-N-(2-hydroxyethyl)acetamide	-6.88	1001 glutamic acid, 990 tyrosine and 991 tyrosine
 N-(2-methyl-7-nitro-1H-benzimidazol-6-yl)acetamide	-7.8	13265 serine
 5-[(4,5-dioxo-3,4,5,6-tetrahydropyrido[3,4-d]pyridazin-1-yl)methyl]-3H-furanium	-7.94	1736 phenylalanine

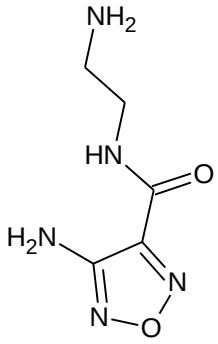
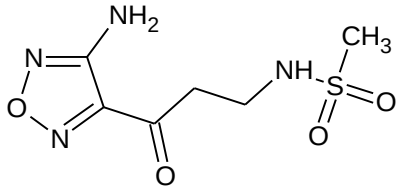
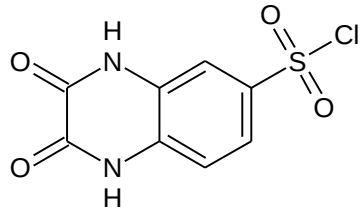
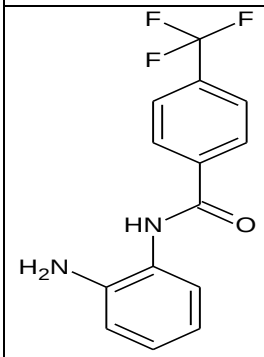
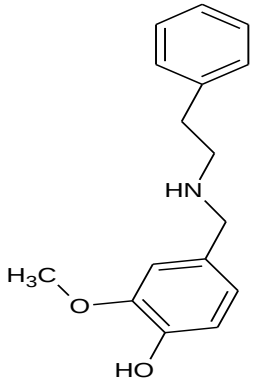
 2,6-dihydroxypyridine-4-carboxylic acid	-8.4	882 isoleusine, 885 valine and 886 proline
 4-(4-hydroxybenzyl)-5-methyl-1,3-dihydro-2H-imidazole-2-thione	-9.8	1052 glysine and 943 lysine
 4-amino-1H-pyrazole-5-carbohydrazide	-8.5	residue 1787
 2-[(3,5-dinitrophenyl)sulfanyl]ethanol	-8	1103 glysine
 4'-nitro-1H,1'H,1''H-4,3':5',4''-terpyrazole	-8	957 glysine, 949 threonine, 964 threonine, 1300 aspartate and 1375 cysteine

 ethyl 7-amino-3-cyanopyrazolo[1,5-a]pyrimidine-6-carboxylate	-7.4	552 lysine and 551 aspartate
 N-[2-nitro-4-(trifluoromethyl)phenyl]ethane-1,2-diamine	-8.4	1779 HEM
 5-methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidine-6-carboxylic acid	-6.1	1300 aspartate
 3-(1H-tetrazol-1-yl)phenol	-5.6	1194 ASN and 1102 alanine

 3-[4-(prop-2-en-1-yl)-5-sulfanyl-4H-1,2,4-triazol-3-yl]phenol	-8.12	1332 glysine, 1194 ASN and 1102 alanine
 1H-pyrazol-5-ylmethanol	-6.47	1106 arginine and 1103 glysine
 7-hydroxy-1,5,6,7-tetrahydro-4H-imidazo[4,5-d]pyridazin-4-one	-6.8	1326 serine, 982 glysine, and 989 glysine
 2-amino-5-(4-hydroxybenzyl)-4-methyl-1H-pyrrole-3-carbonitrile	-9.6	612 glysine and 565 glutamic acid
 2-methyl-5-(1H-tetrazol-1-yl)phenol	-8.7	466 proline
 3-hydroxy-4a,8a-dihydropyrimido[4,5-	-7.4	1779 HEM and 1225 aspartate

e][1,2,4]triazine-6,8(5H,7H)-dione		
 3-hydroxy-4-(2-hydroxyethyl)-1H-pyrazole-5-carboxylic acid	-8.2	1300 aspartate, 959 isoleusine, 956 leusine, 949 threonine and 964 serine
 4-{[hydroxy(phenyl)methyl]amino}tetrahydrothiophene-3-ol	-9.4	1038 proline
 2-amino-4,7-dioxo-3,4,5,6,7,8-hexahydropyrido[2,3-d]pyrimidine-5-carboxylic	-9.1	110 cysteine, 482 ASN, 116 isoleusine, 117 glysine and 460 aspartate
 (4-fluorophenyl)(piperidin-4-yl)methanol	-9.39	906 serine
	-10.3	1779 HEM

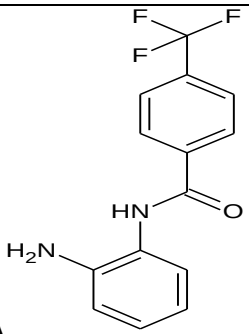
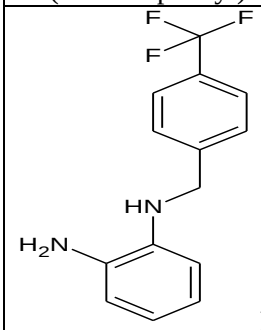
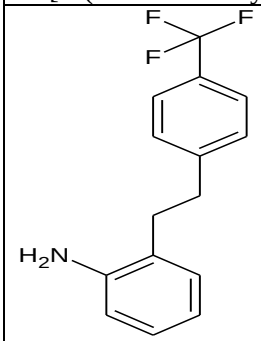
4-amino-N-(2-phenylethyl)benzenesulfonamide		
 7-(trifluoromethyl)-3,4-dihydroquinoxalin-2(1H)-one	-8	885 valine and 882 isoleusine
 N-(1-phenylethyl)benzenesulfonamide	-9.5	680 tryprophan and 774 ASN
 5-nitro-N-[2-(thiophen-2-yl)ethyl]pyridin-2-amine	-9.3	none
 5-(phenylcarbamoyl)imidazolidine-4-carboxylic acid	-10.2	1779 HEM
 2,2'-[(4-hydroxybenzyl)imino]diacetic acid	-7.3	1332 glysine and 1779 HEM
 4-nitro-N-(2-nitrophenyl)-4,5-dihydro-1H-pyrazole-3-carboxamide	-8.3	1122 ALA

 4-amino-N-(2-aminoethyl)-1,2,5-oxadiazole-3-carboxamide	-8.9	1300 aspartate
 N-[3-(4-amino-1,2,5-oxadiazol-3-yl)-3-oxopropyl]methanesulfonamide	-7.3	methionine and cysteine
 2,3-dioxo-1,2,3,4-tetrahydroquinoxaline-6-sulfonyl chloride	-7.8	993 serine and residue 17225
 N-(2-aminophenyl)-4-(trifluoromethyl)benzamide	-10.7	885 valine
 2-methoxy-4-{[(2-phenylethyl)amino]methyl}phenol	-10.55	885 valine

Ligand Modification to Improve Pose Energy

Two ligands with a ligandscore of -10 kcal/mol were chemically modified to improve their binding affinity to the enzyme. As a result several derivatives with pose energies below -11 were obtained with lowest pose energy being -15.3 kcal/mol. These results are as shown in the Table 6 and 7

Table 6. Modifications of N-(2-aminophenyl)-4-(trifluoromethyl)benzamide (A)

Ligand structure	**Best pose energy (kcal/mol)**	**Residues with which it forms hydrogen bonds**
 **A** N-(2-aminophenyl)-4-(trifluoromethyl)benzamide	-10.7	885 valine
 **B** N-[4-(trifluoromethyl)benzyl]benzene-1,2-diamine	-11.4	959 isoleusine
 **C** 2-{2-[4-(trifluoromethyl)phenyl]ethyl}aniline	-11.6	1739 glysine and 1346 arginine

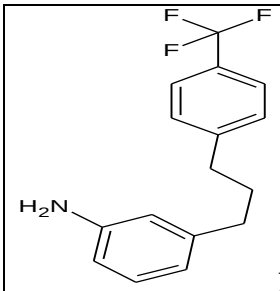
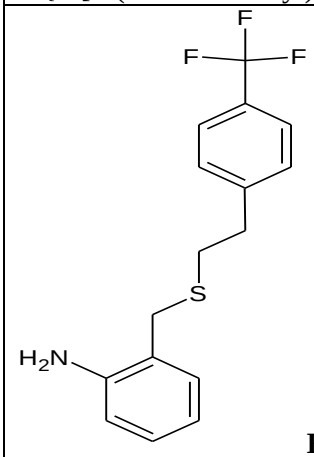
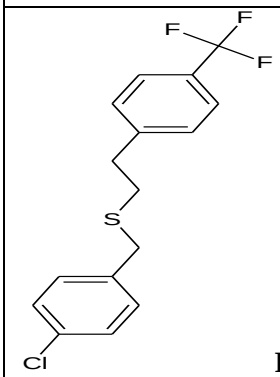
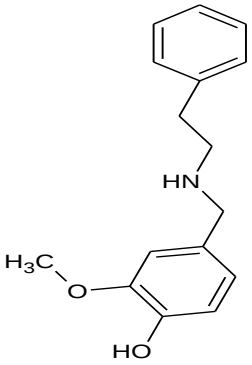
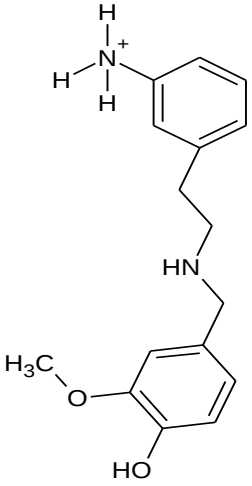
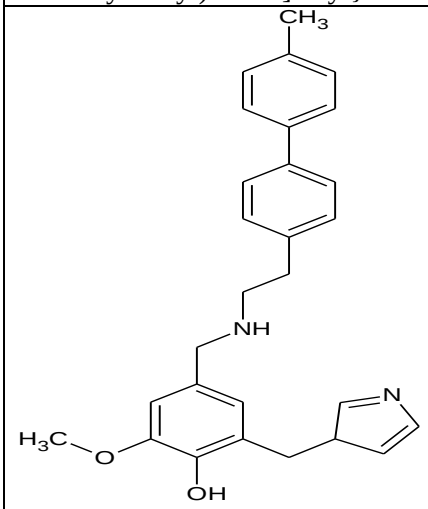
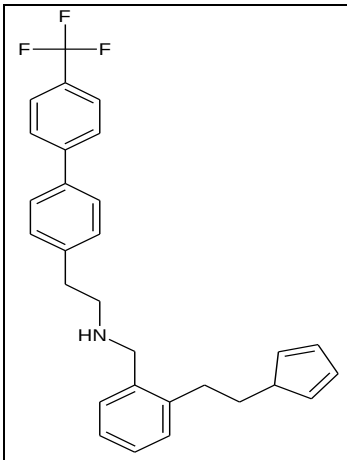
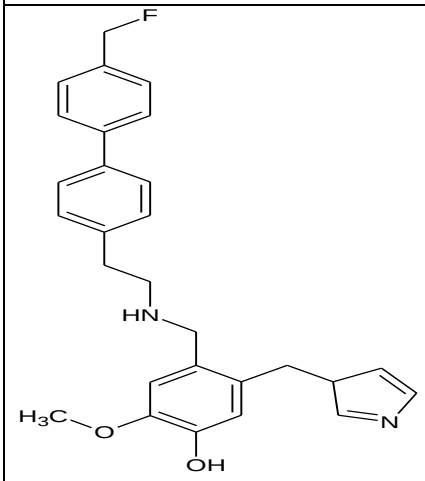
 **D** 3-{3-[4-(trifluoromethyl)phenyl]propyl}aniline	-12.8	none
 **E** 2-[({2-[4-(trifluoromethyl)phenyl]ethyl}sulfanyl)methyl]aniline	-12.7	none
 **F** 1-chloro-4-[({2-[4-(trifluoromethyl)phenyl]ethyl}sulfanyl)methyl]benzene	-13.46	none

Table 7. Modifications of 2-methoxy-4-{[(2-phenylethyl)amino]methyl}phenol (G)

Ligand structure	**Best pose energy (kcal/mol)**	**Residues with which it forms hydrogen bonds**
 **G** 2-methoxy-4-{[(2-phenylethyl)amino]methyl}phenol	-10.55	885 valine
 **H** 3-{2-[(4-hydroxy-3-methoxybenzyl)amino]ethyl}anilinium	-11.4	956 isoleusine
 **I**	-15.3	885 valine, 882 isoleucine, 1773 HEM and 3435 residue

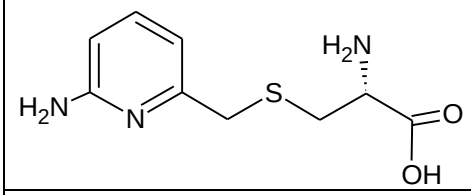
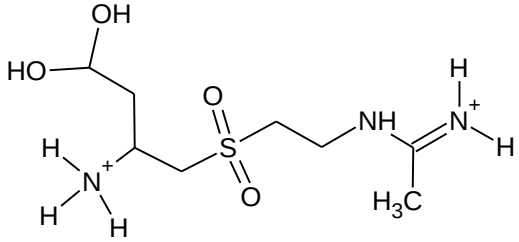
 **J**	-14.07	529 threonine
 **K**	-14.88	1773 HEM

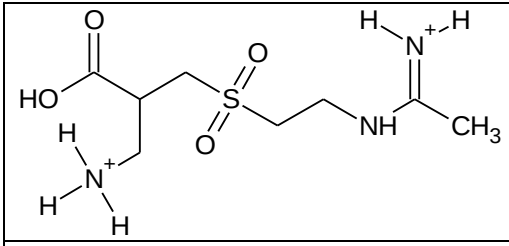
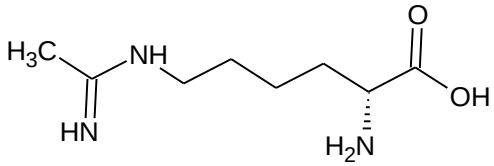
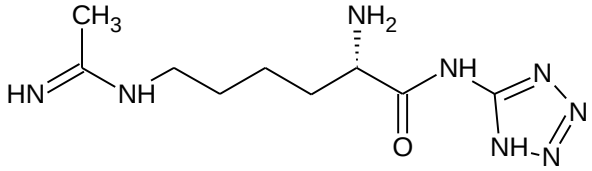
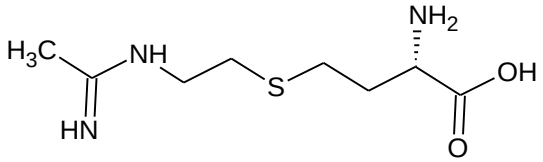
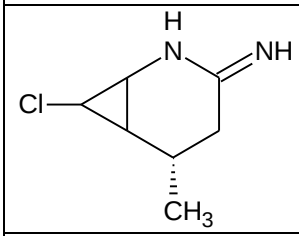
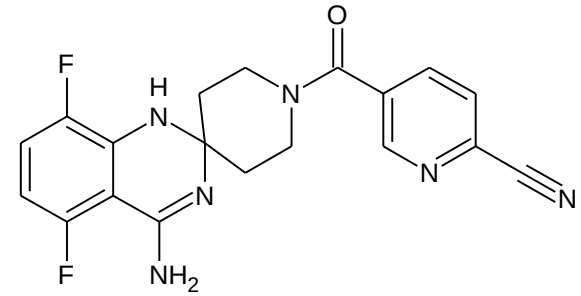
G is the parent compound while H,I,Jand K are the results of modifications of G

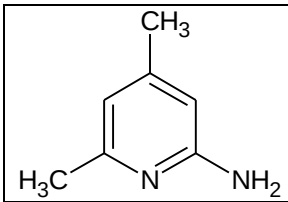
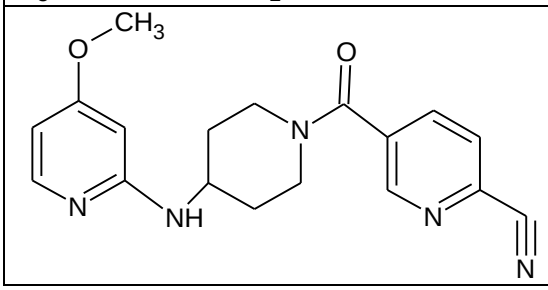
Docking of Inhibitors found in Literature Review

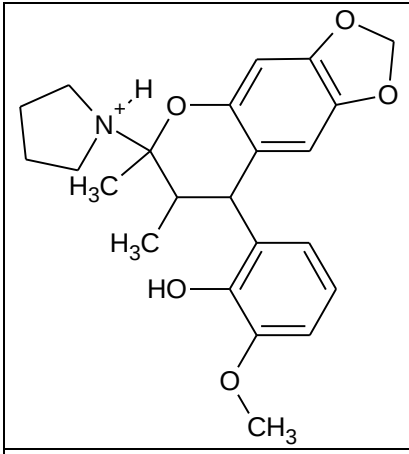
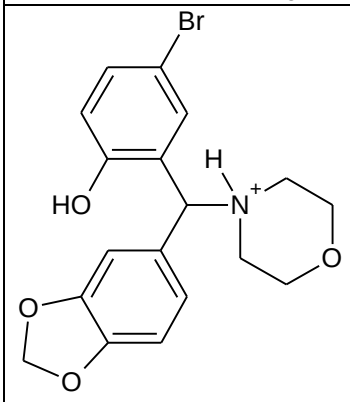
Results of the docking of inhibitors reported in various literature reviewed in this study are shown in the following Table 8.

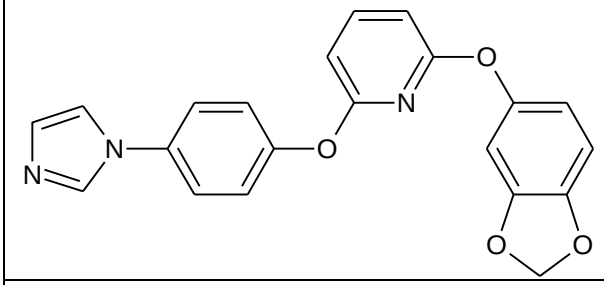
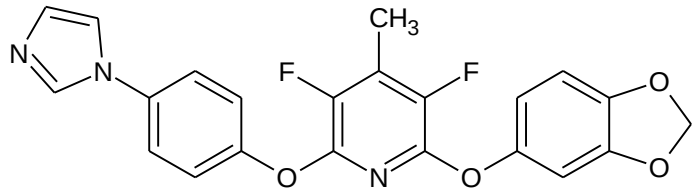
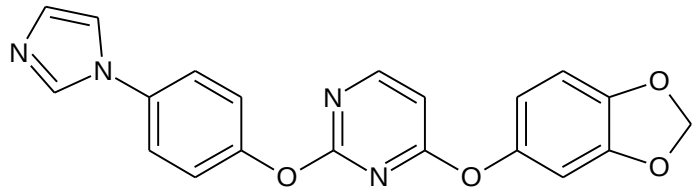
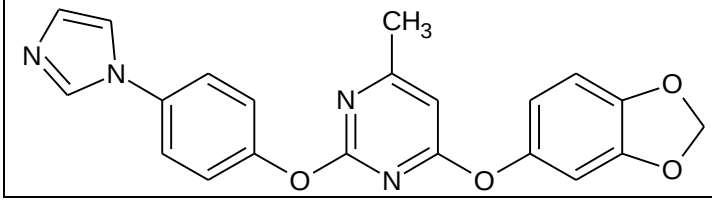
Table 8. Inhibitors in literature review and their pose energy

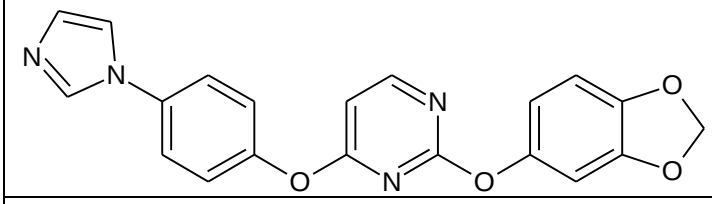
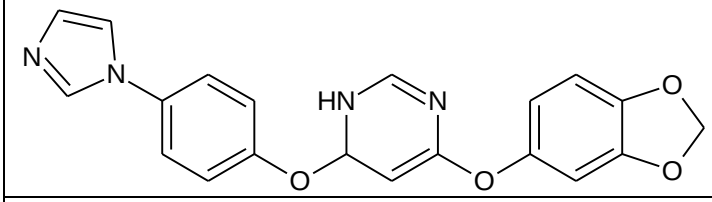
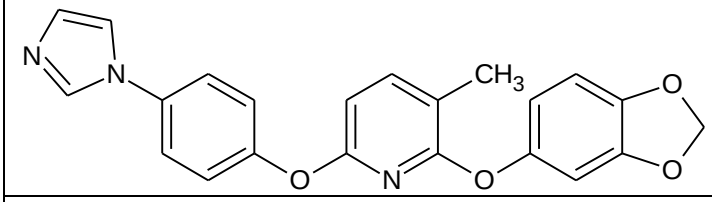
Inhibitor	**Best pose energy (Kcal/mol)**
	-9.93
	No acceptable pose

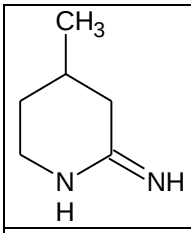
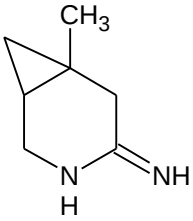
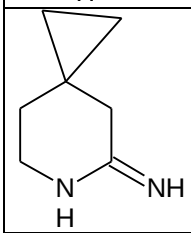
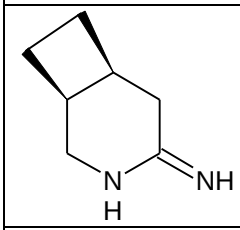
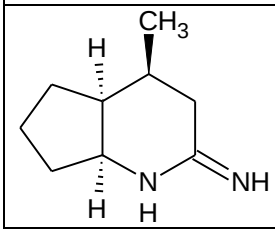
	-7.7
	-5.95
	-6.50
	-4.98
	-8.66
	No acceptable pose

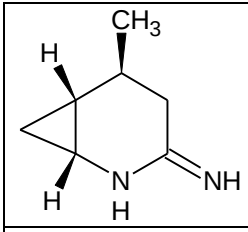
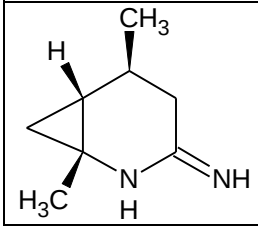
	-8.64
	-8.14

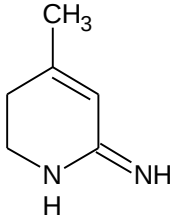
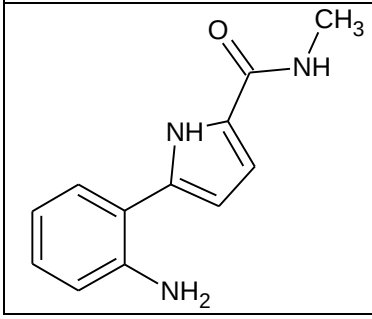
	-8.45
	-9.397

	-8.76
	-8.34
	-8.42
	-8.13

	-8.30
	-8.17
	-8.14

	-8.84
	-9.02
	-8.60
	-9.25
	-9.39

	-9.47
	-8.64

	-8.8
	-9.38

DISCUSSION

This section first summarizes the findings and then compares the NOS binders identified in this project with the natural ligand tetrahydrobiopterin and inhibitors found in literature. The reason why some of the identified binders are promising in treating stroke is also explained.

After docking of ligands identified through virtual screening, five compounds were found to have pose energy ranging from -10.2 to -10.7 kcal / mol (Table 5). Modification of two ligands that were found to have lowest pose energy resulted into nine derivatives with pose energy between -11.4 and -15.3 kcal / mol as shown in Table 6 and 7. Of all compounds docked against the binding site of tetrahydrobiopterin in nitrogen oxide synthase, 4-({[2-(4'-methylbiphenyl-4-yl)ethyl]amino}methyl)-2-methoxy-6-(3H-pyrrol-3-ylmethyl)phenol (Figure 10) had the lowest binding energy, with a pose energy of -15.3 kcal /mol.

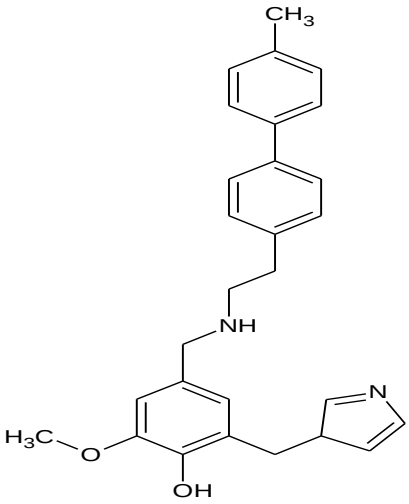


Figure: 10: 4-({[2-(4'-methylbiphenyl-4-yl)ethyl]amino}methyl)-2-methoxy-6-(3H-pyrrol-3-ylmethyl)phenol.

Graphics related to the above named compound are presented in Figure 11. Figure 12 is a depiction of residues that form hydrogen bonds with the best binding ligand while Dock calculation for the best binding ligand are displyed in Figure 13.

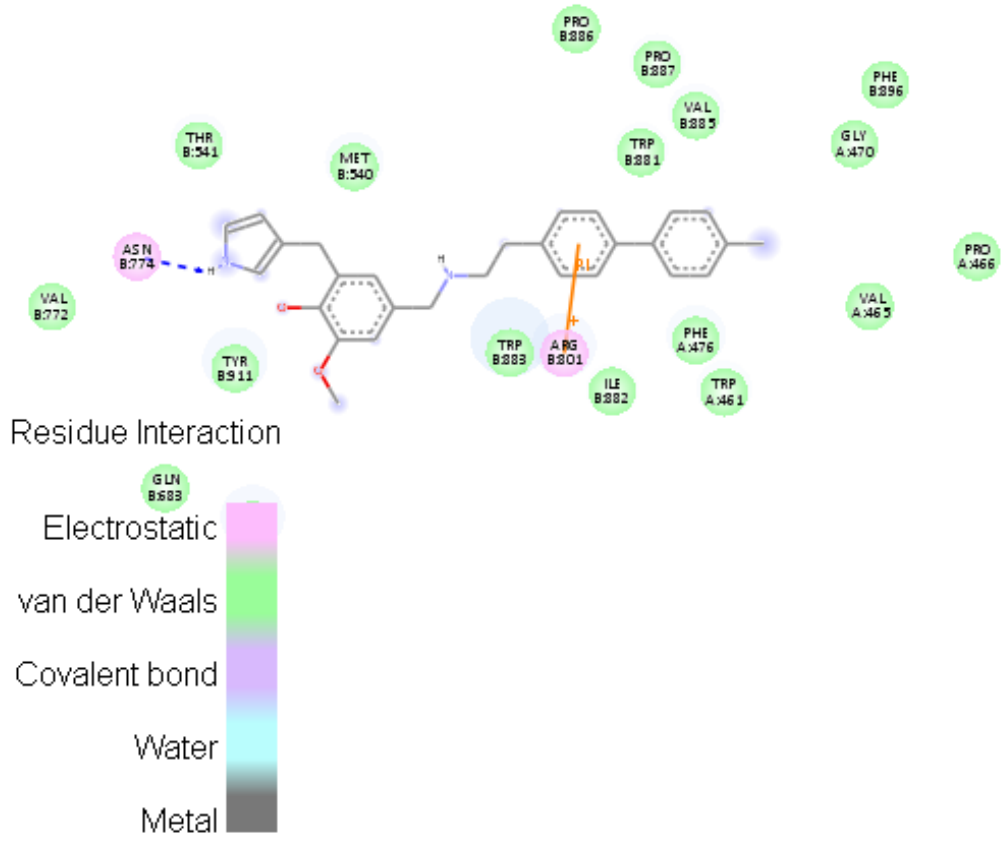


Figure 11: Interaction between the best binding ligand and its receptor.

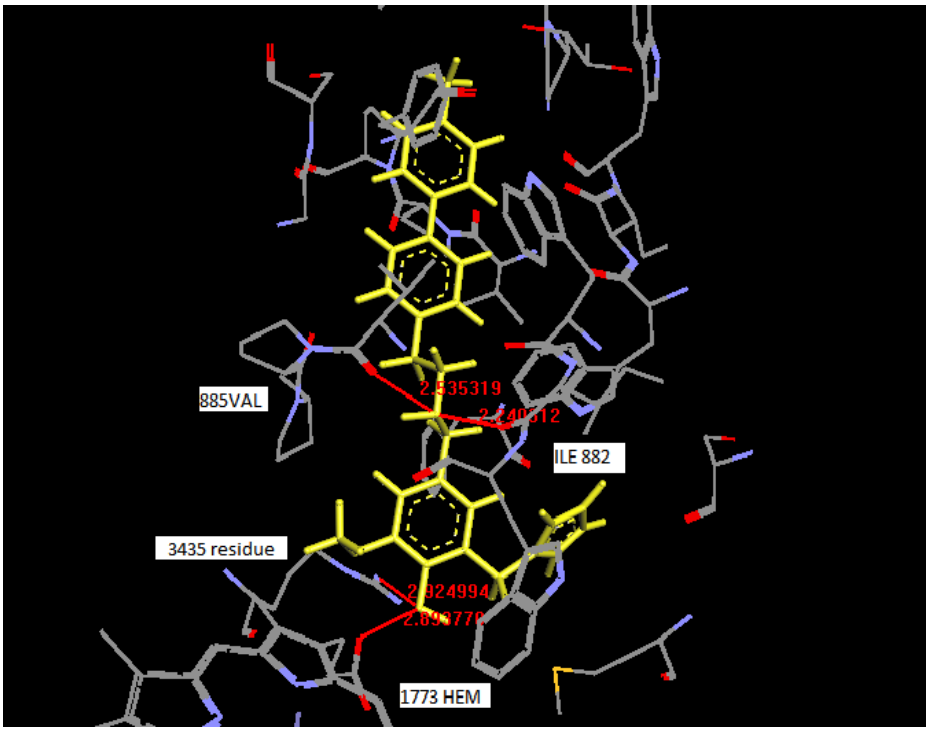


Figure 12: Residues that form hydrogen bonds with the best binding ligand.

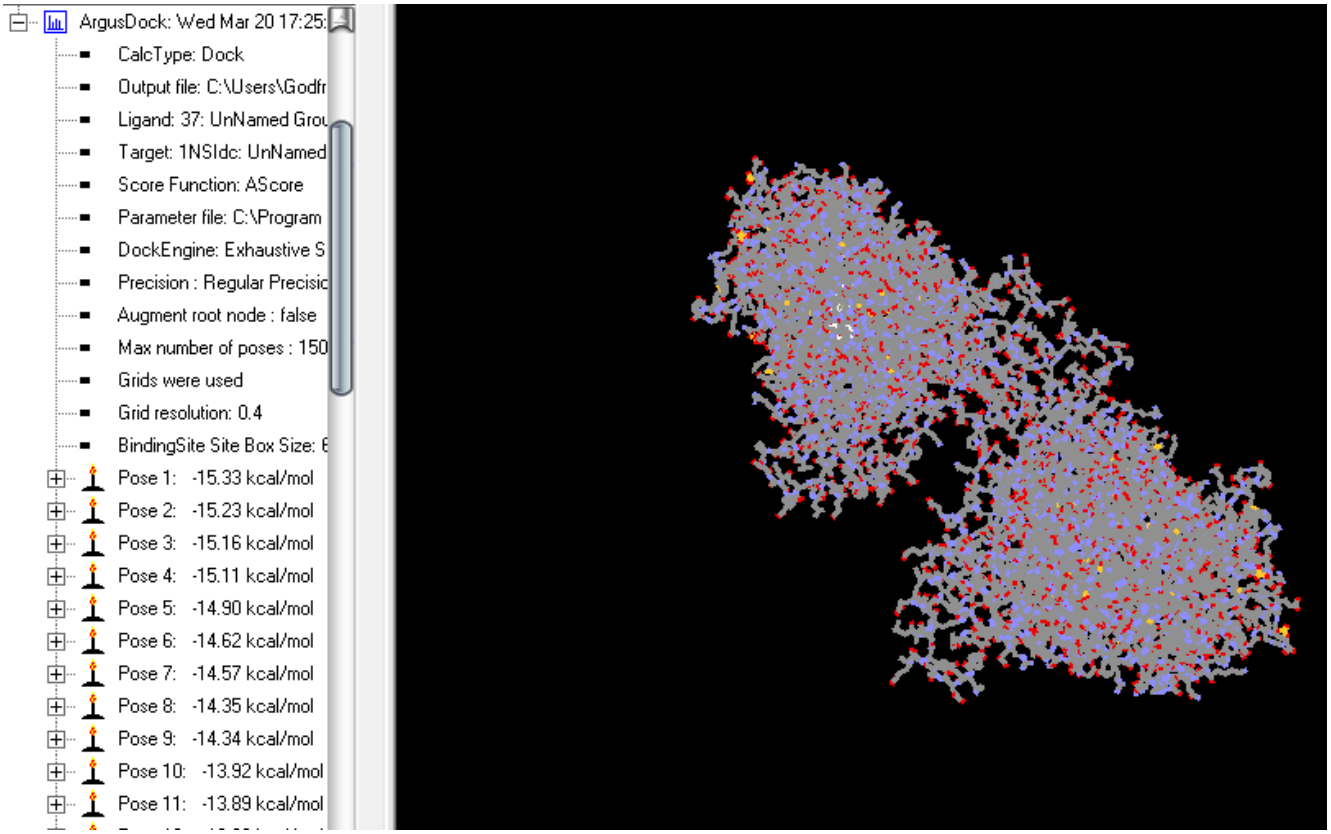


Figure 13: Dock calculation for the best binding ligand.

The compound with the second best pose energy was 4-({[2-(4'-fluoromethylbiphenyl-4-yl) ethyl] amino} methyl)-2-methoxy-6-(3H-pyrrol-3-ylmethyl) phenol whose structure and graphics are presented in Figure 14. Its pose energy was -14.88 kcal / mol., while its Dock calculations are depicted in Figure 15. Figure 16 shows the interaction of 773 HEM with 4-({[2-(4'-fluoromethylbiphenyl-4-yl)ethyl]amino}methyl)-2-methoxy-6-(3H-pyrrol-3-ylmethyl)phenol through hydrogen bonding.

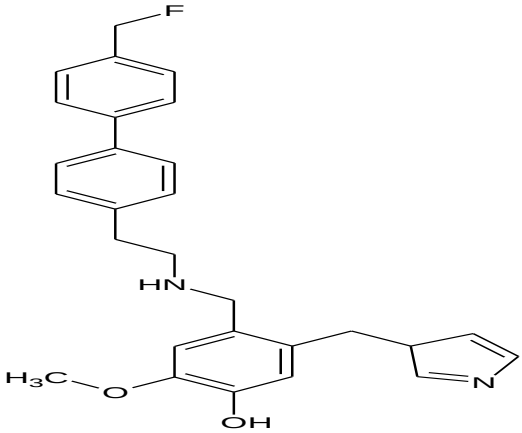


Figure 14: 4-({[2-(4'-fluoromethylbiphenyl-4-yl)ethyl]amino}methyl)-2-methoxy-6-(3H-pyrrol-3-ylmethyl)phenol

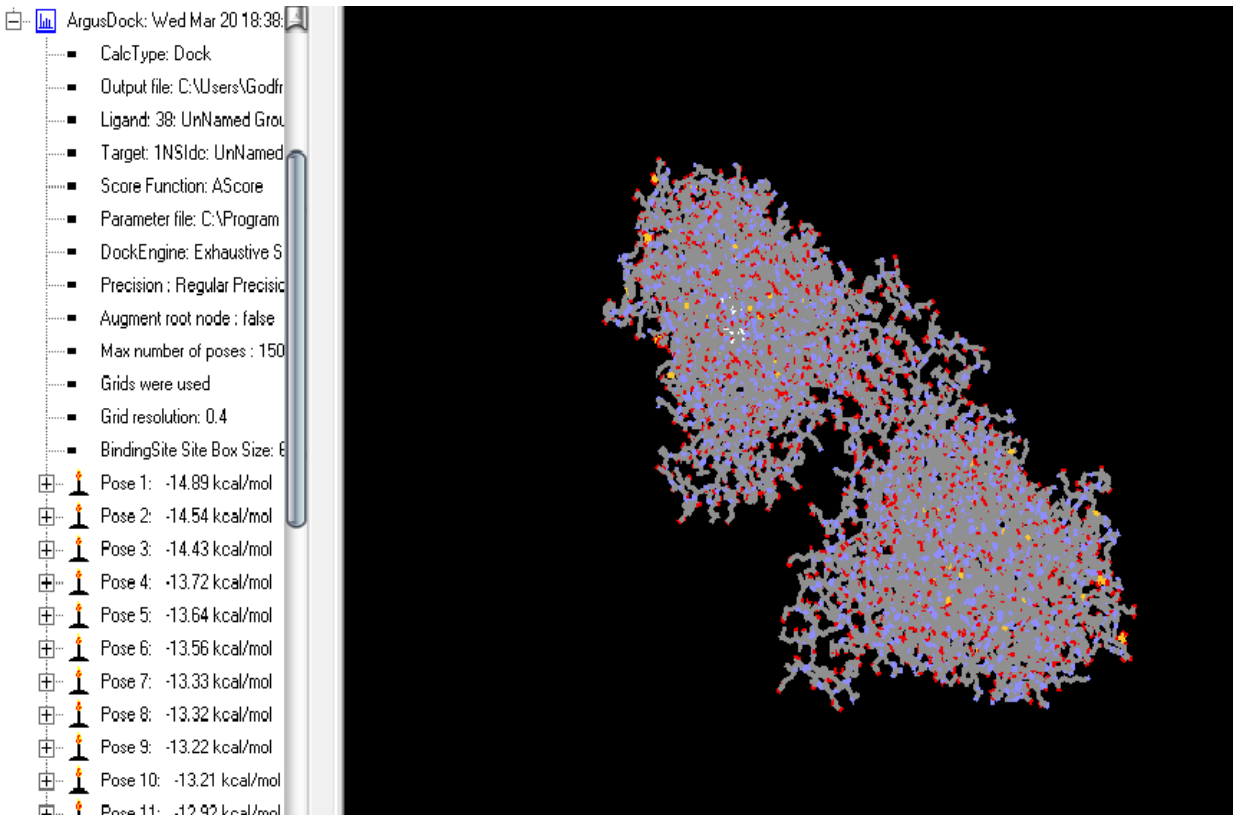


Figure 15: Dock calculation for 4-({[2-(4'-fluoromethylbiphenyl-4-yl)ethyl]amino}methyl)-2-methoxy-6-(3H-pyrrol-3-ylmethyl)phenol.

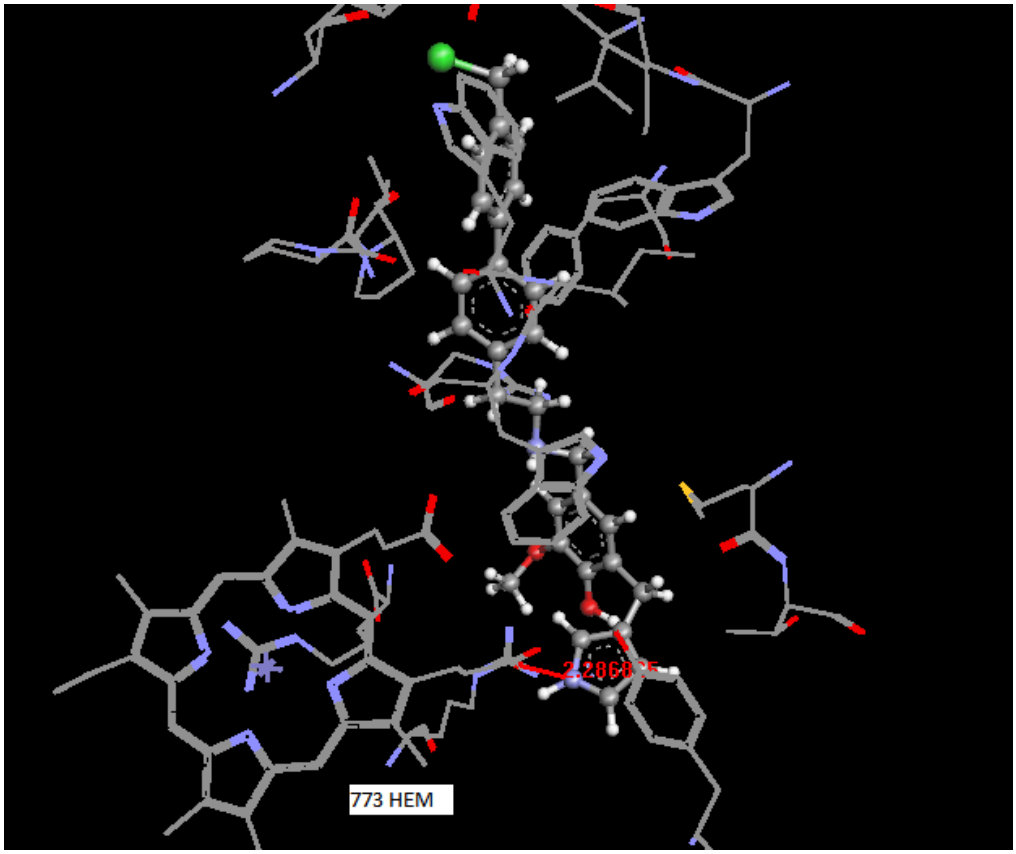


Figure 16: Interaction of 773 HEM with 4-({[2-(4'-fluoromethylbiphenyl-4-yl)ethyl]amino}methyl)-2-methoxy-6-(3H-pyrrol-3-ylmethyl)phenol through hydrogen bonding.

The nine best binding ligands (ligand B- E and H-K in Table 6 and 7) obtained in this study have pose energies which are well below that of tetrahydrobiopterin, the natural ligand of nitrogen oxide synthase. The pose energies of these ligands are below -11 (the lowest being -15.3) while that of tetrahydrobiopterin is -7kcal/mol. Since pose energy is a measure of affinity a ligand to a receptor, these ligands are expected to have high affinity to NOS than tetrahydrobiopterin (Kitchen et al., 2004). Pose energies of Ligands B- E and H-K were also lower than those of inhibitors 1-27 (Table 8) as reported in various literatures that were reviewed in this project. During the project 27 inhibitors were docked and their pose energies ranged from -4.98 to -9.93 kcal / mol. Two of the inhibitors in literature (inhibitor 3 and 8) had no acceptable pose.

Both ligand I and K identified in this project are capable of forming hydrogen bonds with HEM of NOS . This increase their potential as inhibitors of NOS because by binding HEM they interfere with its ability to participate in the electron transfer which is an essential part of the NOS mediated production oof NO as shown in Figure 17.

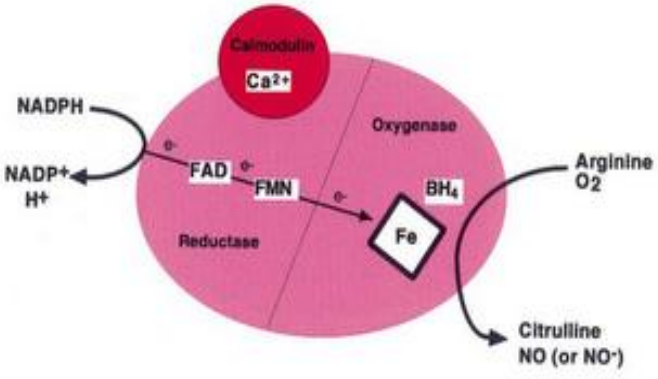


Figure 17: NOS mediated production of NO (Alderton et al., 2005).

HEM is also essential for the binding of L- arginine to NOS (Nishimura et al., 1995). Hence by binding the heme site, ligand I and K have the potential to prevent the binding of amino acid L-arginine to NOS and thus block NO production. The same conclusion was reached by Mayer et al., (1994) who reported that 7-nitroindazole is capable of antagonizing L-arginine and H_4biopterin binding to NOS by binding to the heme site.

CONCLUSION

Through QSAR and docking compounds A-K have been found to have low pose energies which make them potential for use as NOS inhibitors in the management of stroke. Some of these compounds bind heme site of the enzyme which enhance their potential as NOS inhibitors. Despite the excellent binding properties that these compounds have portrayed which testifies their potential as antistroke agents, further studies need to be done to establish their ADMET and toxicity profiles.

REFERENCES

Alderton, W.K., Cooper C.E., Knowles, R.G., (2005). Inhibition of nitric-oxide synthase 2 by aminoguanidine provides neuroprotection of retinal ganglion cells in a rat model of chronic glaucoma . **357**:593-615. Proceedings of the Academy Science of the United States of America.

American Heart Association (2014). Heart disease and stroke statistics-2014 update. Dallas, Texas: American Heart Association. Available at: http://www.heart.org/idc/groups/ahamah-public/@wcm/@sop/@smd/documents/downloadable/ucm_459072.pdf [accessed on June 19, 2014).

Aparna, V., Desiraju, R., GopalakrishnanB., (2007). Insights into ligand selectivity in nitric oxide synthase isoforms: A molecular dynamics study. *Journal of Molecular Graphics and Modelling*. **26**(2):457–470.

Armenian medical network, (2005). What drugs are used to treat stroke patients? , retrieved on April25, 2013 fromhttp://www.health.am/ab/.

Arthur, H., Akira, S., Bernard B., (1999). Inhibition of nitric-oxide synthase 2 by aminoguanidine provides neuroprotection of retinal ganglion cells in a rat model of chronic glaucoma. Proceedings of the National Academy of Sciences of the United States of America *(PNAS)*. **96**(17):9944–9948.

Bano, D, Young, K.W., Guerin C.J., Lefeuvre, R., Rothwell N.J., Naldini, L., Rizzuto, R., Carafoli, E., Nicotera, P., (2005). Cleavage of the plasma membrane Na+2/Ca2+ exchanger in excitotoxicity, *Cell*. **120**:275–285.

Beal, M.F., (1992). Mechanisms of excitotoxicity in neurologic diseases. *Journal of FASEB.* **6:** 3338–3344.

Beauchamp, N. and Bryan, R., (1998). Acute cerebral ischemic infarction: a pathophysiologic review and radiologic perspective. *American Journal of Radiology*. **171**:73-84.

Cacha, P., Chris, E., Richard, G.K., (2002). Neuroprotection by selective nitric oxide inhibition at 24 hours after perinatal hypoxia-ischemia. *Stroke.* **33**:2304- 2310.

Castillo, J., Dávalos, A., Naveiro, J., Noya, M., (1996). Neuroexcitatory amino acids and their relation to infarct size and neurological deficit in ischemic stroke. *Stroke*. **27**:1060-1065.

Eliasson, M.J., Huang, Z., Ferrante, R.J. et al., (1999). Neuronal niric oxide synthase activation and peroxynitrite formation in ischemic stroke linked to neural damage. *Journal of Neuroscience*. **19**:5910-5918.

Fengtian, X., Jinwen, H., Haitao, J., Jianguo F., Huiying, L., Pavel, M., Linda, J., Thomas, L., Richard, B., (2010). Structure-based design, synthesis, and biological evaluation of lipophilic-tailed monocationic inhibitors of neuronal nitric oxide synthase. *Bioorganic and Medicinal Chemistry*. **18**(18):6526–6537.

Ghadeer, A.R.Y., Mayyada, S., Hebah, A., Mutasem, O., (2012). Extensive ligand-based modeling and in silico screening reveal nanomolar inducible nitric oxide synthase (iNOS) inhibitors. *Journal of Molecular Graphics and Modelling*. **37**:1-26.

Goodman, C.C., Fuller, K.S., Boissonnault, W.G., (2003). Pathology: Implications for the Physical Therapist (2nd Edition). Philadelphia, PA: Saunders. Available at: http://www.amazon.com/Pathology-Implications-Physical-Therapist-3e/dp/1416031189 [accessed 19 June, 2014].

Herderscheê, D., Hijdra, A., Algra, A., Koudstaal, P.J., Kappelle, L.J., van Gijn, J., (1992). Silent stroke in patients with transient ischemic attack or minor ischemic. *Stroke*. **23**(9):1220–4.

Jeanie, L.D., (2002). Drugs Stop Stroke Damage,WebMD Health News. Available at: http://www.webmd.com [accessed June 19, 2014].

Jenny, H., (2012). New blood-thinning drug could stop 5,000 strokes a year. Available at: www.dailymail.co.uk [accessed June 19, 2014].

Kitchen, D.B., Decornez, H., Furr, J.R., Bajorath, J., (2004). "Docking and scoring in virtual screening for drug discovery: methods and applications". *Nature reviews. Drug discovery*. **3**(11):935–49.

Lo, E., Moskowitz, M., Jacobs, T., (2005) Exciting, radical, suicidal: how brain cells die after stroke. *Stroke*. 36:189-192.

Love S., (1999). Oxidative stress in brain ischemia, *Brain Pathololog*y. 9:119-131.

Matherset, C.D., Boerma, T., Ma Fat, D., (2009). Global and regional causes of death. *British medical bulletin*, **92**:7–32.

Mayer, B., Klatt, P., Werner, E.R., Schmidt, K., (1994). Neuropharmacology. **33**:1253–1259.

Nakka, V.P., Gusain A., Mehta S.H., Raghubir, R., (2008). Molecular mechanisms of apoptosis in cerebral ischemia: multiple neuroprotective opportunities. *Molecular Neurobiology*. **37**:7-38.

NICE., (2012). New drug for stroke prevention approved by NICE. Available at: www.nice.org.uk [accessed June 19, 2014].

Nishimura, J.S., Martasek, P., McMillan, K., Salerno, J.C., Liu, Q., Gross S.S., Masters, B.S.S., (1995). *Biochem. Biophys. Res. Commun.*, **210**:288–294.

Ryosuke, I., Naoki, H., Tsunehiko, H., (2006). Design, synthesis, and evaluation of new type of l-amino acids containing pyridine moiety as nitric oxide synthase inhibitor. *Bioorganic and Medicinal Chemistry*. **14**(10):3563–3570.

Sims, N.R, Muyderman, H., (2009). "Mitochondria, oxidative metabolism and cell death in stroke". *Biochimica et Biophysica Acta.* **1**: 80-91.

Stanley, J., (2013). Stroke Overview, Incidence and Prevalence. Available: http://www.healthcommunities.com/stroke/overview-of-stroke.shtml [accessed June 19, 2014].

Neurological Disorders and Stroke (2009) *Stroke: Challenges, Progress, and Promise.* **9**:6451 .

Wang, J., (2010). Preclinical and clinical research on inflammation after intracerebralhemorrhage, *Progress in Neurobiology*, **92**(4):463-477.

Warner, D.S., Sheng, H., Batinic-Haberle, I., (2004). Oxidants, antioxidants and the ischemic brain. *J ExpBiol,* **207**:3221-3231.

Wendy, K., (2001). Nitric oxide synthases : structure, function and inhibition. *UK Biochemistry Journal*. **357**:593-615.

Yamashima, T., (2004): Ca2+-dependent proteases in ischemic neuronal death: a conserved 'calpain-cathepsin cascade' from nematodes to primates. *Cell Calcium*, **36**:285-293.