World Journal of Pharmaceutical Sciences
ISSN (Print): 2321-3310; ISSN (Online): 2321-3086
Published by Atom and Cell Publishers
Available online at: http://www.wjpsonline.com/
Research Article

Clinical Study on Serum Nitric oxide levels in Osteoarthritis and Structure-Based Drug Design of New Inducible Nitric Oxide Synthase inhibitors

Sadia Ikhlaque Sheikh[1], Ambreen Hafeez[2], Ejaz Ahmed[3]

[1]Department of Biochemistry and [2]Biophysics/Molecular modeling Research Unit, Department of Biochemistry, Dow International Medical College, Dow University of Health Sciences, Karachi, Pakistan, 75270
[3]United Medical and Dental College, Korangi creek, Karachi, Pakistan, 75190

Received: 21-11-2013 / Revised: 06-12-2013 / Accepted: 10-01-2014

ABSTRACT

Osteoarthritis (OA) is an old age disease and caused by both biochemical and mechanical factors. Nitric oxide (NO), is a metabolic product of L-arginine produced by nitric oxide synthase (NOS). NO and its derivatives were found to have a number of different functions in both normal and pathophysiological joint conditions. According to recent studies excessive production of NO by excessive iNOS (inducible nitric oxide synthase) stimulation is responsible for several pathological conditions including OA. We conducted clinical study on 150 female patients suffering from OA with age group 45 – 65 years (mean age 55.5 years) to find out whether the levels of nitric oxide are associated with OA. Nitrite levels were measured in the serum as marker of nitric oxide (NO) production by assay method based on Griess assay method. In OA patients serum nitrite levels were much higher (116.8µmol/L) as compared to control subjects (48.6µmol/L) ($p<0.001$). At the end of the clinical study we conducted structure based drug designing (molecular docking study) for the *in silico* search of new iNOS inhibitors as it is involved in the excessive formation of NO which ultimately cause osteoarthritis.

Key Words: Osteoarthritis, Nitric oxide, Nitric oxide synthase inhibitor.

INTRODUCTION

Nitric oxide (NO) is a free radical produced in mammalian cells during the metabolism of L-arginine by NO synthase (NO). This reaction involves one of the three isoforms of nitric oxide synthase (NOS). Two of the NOS enzymes, namely endothelial NOS (eNOS) and neuronal NOS (nNOS), are calcium dependent and constitutively produce relatively low levels of NO. The inducible isoform (iNOS) is expressed for a longer period of time upon activation by a variety of factors, including the inflammatory cytokines TNF-α and lipopolysaccharide reviewed by Weinberg and co-workers [1]. Once synthesized, NO can diffuse within the same cell or neighbouring cells, where it binds to the heme group of soluble guanyl cyclase to generate cGMP from GTP [2]. Activated cGMP then binds specifically to target proteins including transcription factors, protein kinases and phosphodiesterases to elicit downstream effects. However, NO can also act in a cGMP-independent manner, for example by directly modifying proteins or contributing to the oxidation of proteins and lipids, and thus increasing the complexity and number of potential roles of NO in normal and pathological conditions [3].

NO and its derivatives play critical roles in the production of nociception and pain, which is the primary cause of functional disability in OA. Mechanical loading of bone and isolated bone cells cause a rapid and transient release of NO. Different data suggest that in addition to osteoblasts, osteocytes may be another cellular source of NO production [4] and it is primarily a catabolic factor in osteoarthritis (OA). NO may possess some beneficial effects on other cell types, including tendons and osteoblasts, which could also potentially be present in chondrocytes [5] . Therefore further investigations are needed to find out the positive role of NO in bone disorders. There is a wide range of iNOS inhibitors described in the literature and went through clinical trials but due to some limitations, including toxicity, poor

**Corresponding Author Address: Dr. Sadia Ikhlaque Sheikh, Assistant Professor, Department of Biochemistry, Dow International Medical College, Dow University of Health Sciences, Karachi, Pakistan, 75270, Tel : +92-3333341025, Email: drsadia666@hotmail.com*

bioavailability or poor selectivity, there is a continuous interest for the investigation and development of new iNOS [6] The more recent effort in this respect is the use of *in silico* tools such as structure-based drug designing for the development of new iNOS.

The present study focused on both; the clinical investigations to confirm the potential role of NO in osteoarthritis (OA) due to over expression of iNOS and *in silico* search for new iNOS inhibitors through structure-based drug design (molecular docking study).Molecular docking is an *in-silico* tool to simulate processes where a ligand position is determined in a predefined binding site in the receptor protein molecule mainly the enzyme .

CLINICAL STUDY

Materials and Method: The study was conducted on 150 female patients suffering from OA with age group 45 – 65 years (mean age 55.5 years). The patients were selected from Civil hospital and Dow University of Health Sciences, Karachi, Pakistan on the basis of signs, symptoms, history and severity of disease at joint site and X-Ray of the joints. Patients taking any hormone replacement therapy (HRT), non steroidal anti inflammatory drugs (NSAID), having metabolic disease, rheumatoid arthritis (RA), joint, systemic lupus erythromatosis (SLE) were excluded from the study. Every one filled out the questionnaire giving information about their gender, age, past health problems, present medication, menstrual state and age of menopause. Those with uncertain menstruation history were excluded from the study. The blood specimens were collected and the study was performed in accordance with ethical standards. Permission was given by the Civil Hospital and Dow University of Health Sciences, Karachi. Control group consisted of hundred healthy female subjects (age between 40 to 60 years). Blood sample were drawn and serum was immediately frozen at -70^0C until the analysis was carried out. Nitrite levels were measured as a marker of NO production by direct Griess reaction, which is the simplest and most commonly used assay method [7] Kit was provided by assay designs, 800 Technology Drine, Amn Arbor, Michigan USA). The kit provides the total determination of both nitric oxide products in the sample by conversion of the entire sample nitrate into nitrite, followed by the determination of the total concentration of nitrite in the sample.

Results and Discussion: Table 2 shows the nitrite levels in control and OA subjects ($p<0.001$). Statistical significance among three groups was done by student t- test using SPSS-16 version of statistical software. Results indicate that NO and its derivatives play critical roles in the production of nociception and pain, which is the primary cause of functional disability in OA [8]. Elevated levels of markers of nitric oxide (NO) production are found in osteoarthritic joints suggesting that NO is involved in the pathogenesis of osteoarthritis (OA). In OA, NO mediates many of the destructive effects of interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-alpha) in the cartilage, and inhibitors of NO synthesis have demonstrated retardation of clinical and histological signs and symptoms in experimentally induced OA and other forms of arthritis. There is much evidence indicating the potential role of NO in the pathogenesis of arthritis [9,10].

IN SILICO STUDY

Materials and Method: In the present work, structure-based drug design of iNOS has been carried out to find out the new possible iNOS inhibitors by molecular docking study. Inhibitors were selected from recently published literature [6] and tested against iNOS. The 2D structures of these inhibitors were drawn manually using ACD Lab Chemsketch software and were saved in MDL-mol file format. The structures were then imported into a docking software the Molegro Virtual Docker (MVD) where they were converted to 3D structures and energy was minimized by PLP (piece wise linear potential) force field implemented in MVD. The structures were then utilized for docking study. MVD handles all aspects of the docking processes from preparation of the molecule to determination and charges and protonation states are automatically assigned in combination with the automated prediction of cavities [11]. The X-ray 3-D crystallographic structure of iNOS (PDB code: 1QW4) was extracted from the Protein Data Bank (PDB) [12] (http://www.rcsb.org) present in complex with co- crystallized ligand N-omega-propyl-L-arginine. Docking studies were performed on iNOS enzyme and the selected inhibitors according to standard protocol implemented in MVD 2013.6.0.

At the end of each docking run, interactions are shown in the form of "poses" with the energy values given as MolDock score kcal cal/mol for each pose. 10 independent runs were conducted with the guided differential evolution algorithm of MVD and one pose was returned for each run. The most stable pose was selected according to the best MolDock score predicted by MolDock [Grid] scoring function and re-rank score. Only the best conformation, with highest binding affinity i.e. the lowest binding energy has been chosen for the protein-ligand interaction study.

Results and Discussion: The 2D structures of iNOS inhibitors are given in Table 2.The list of binding energies obtained for each iNOS inhibitor is given in Table 3. Results representing hydrogen bonds formed between the best iNOS inhibitor and iNOS is given in Table 4. X-ray crystal structure of iNOS in complex with co-crystallized ligand (N-omega-propyl-L-arginine) is given in figure 1. Superimposition of iNOS inhibitors with co-crystallized ligand is given in figure 2. Docking of the top ranked inhibitors (inhibitor no 3 and 2 in table **2**) for the studied protein target i.e. iNOS are shown in Figures 3 and 4 respectively. The parameters such as hydrogen bond interactions, lowest scoring values and binding orientations of the docked inhibitors in the enzyme's active site contribute to the biological functioning of compounds. The docking results showed that the best binding affinity of iNOS was found with inhibitor 3 with the lowest MolDock score of -115 kcal/mol and the re-rank score of -100.83 kcal/mol. This can be identified as a competitive type of inhibitor as it made hydrogen bonds with the substrate binding amino acid residues of the enzyme's active site such as Glu 371, Tyr 367 at a distance of 2.45 and 2.66 Å respectively and to some extent to Asp 367.

Inhibitor 2 also showed good MolDock score and re-rank score but did not replace the substrate binding amino acid residues of the enzyme's active site. Instead, it made hydrogen bond with only one amino residue of the binding site i.e. Tyr 367 at a distance of 2.71Å. This may be due to different binding orientation. The remaining inhibitors did not show any successful hydrogen bond formation and binding orientation.

CONCLUSION

Inducible nitric oxide synthase has been investigated as the enzyme responsible for the onset of variety of diseases which can lead to the discovery of new iNOS inhibitors. These studies implicate the NO pathway in the pathogenesis of an inflammatory arthritis and demonstrate the ability of NOS inhibitors to modulate the disease. The present study appear to demonstrate the beneficial effect of blockade of the NO pathway in destructive arthritic lesions and inhibitors selective for iNOS may prove useful in the long-term treatment of osteoarthritis and perhaps other inflammatory diseases.

Table 1: Serum nitrite levels in controls and osteoarthritis patients.

	Controls	patients
Serum nitrite levels (µmol/L)	48.6 ± 1.1 (100)	116.8* ± 1.2 (150)

P< 0.001

values are mean ± S.D

Table 2: Optimized structures of iNOS inhibitors by ACD Lab Chemsketch used as ligands

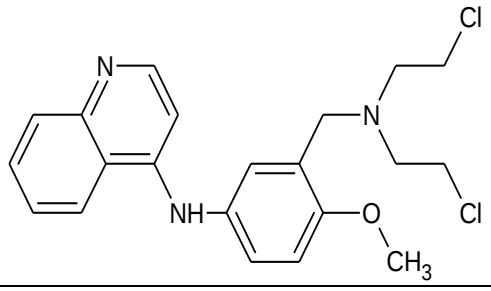
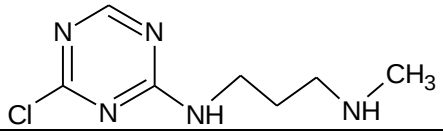
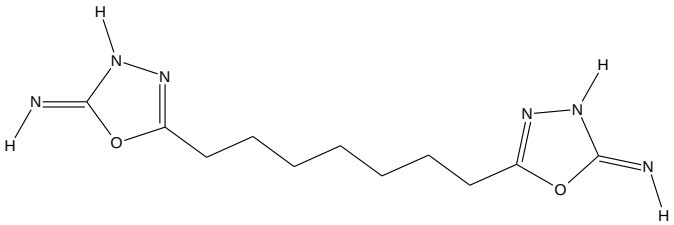
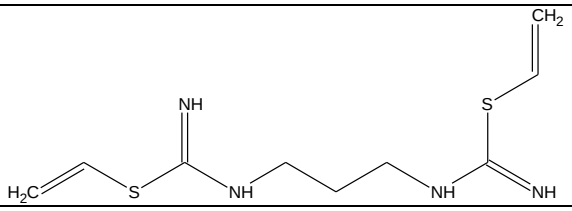
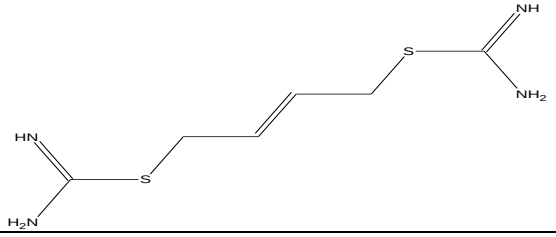
S. No	Ligands	Structure
1	NCI0225379	
2	NCI231540	
3	NCI382943	
4	NCI507481	
5	NCI0014523	

Table 3: Binding Energies Calculated for iNOS inhibitors with the Enzyme inducible Nitric Oxide Synthase by MVD with Different Scoring Parameters

S. No	iNOS inhibitors (Ligands)	MolDock Score (kcal mol^{-1})	Rerank Score (kcal mol^{-1})	H bond Score (kcal mol^{-1})
1	NCI0225379	-92.76	-72.39	-10.75
2	NCI231540	-107.13	-99.62	-3.10
3	NCI382943	-115.39	-100.83	-2.21
4	NCI507481	-97.27	-88.73	-11.31
5	NCI0014523	-90.34	-61.14	-3.51

Table 4: List of H- bonds formed between Top Ranked iNOS inhibitors and Inducible Nitric Oxide Synthase Enzyme

S.No	Enzyme	iNOS inhibitors	Ligand atom	Amino acid residue atom	H-bond distance (Å)	No of hydrogen bonds
1	Nitric Oxide Synthase	NCI382943	NH NH	Glu 371 (O) Tyr 367 (O)	2.45 2.66	2
2		NCI231540	NH	Tyr 507 (O)	2.71	1

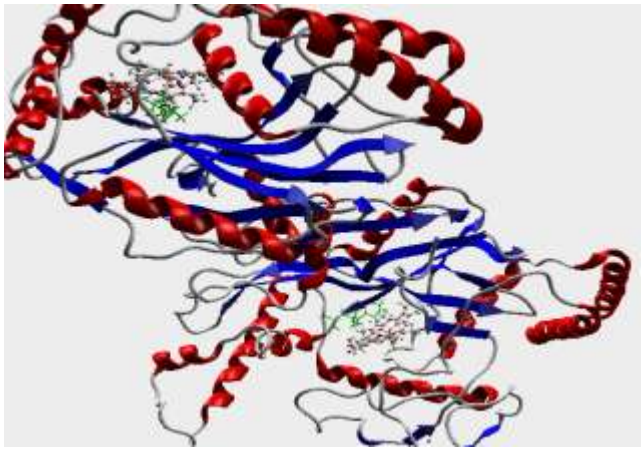
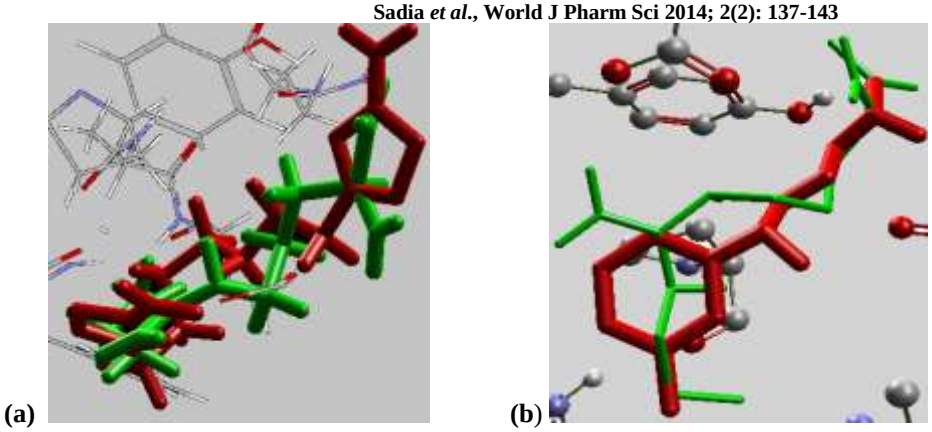


Figure 1: X-ray crystal structure of iNOS (PDB code:1QW4). Beta sheets are shown in blue, Alpha helices in red and bound ligand (N-omega-propyl-L-arginine) in green stick model. a redox cofactor, (6R)-5,6,7,8-tetrahydro-L-biopterin (H4B) and heme are seen in ball and stick in element colors.



(a) **(b)**

Figure 2: Superimposition of iNOS inhibitors with co-crystallized ligand N-omega-propyl-L-arginine (seen in green thick stick model) in X-ray crystal structure of inducible nitric oxide synthase (PDB code: 1QW4). Inhibitors are seen in red thick stick (a) NCI382943 (b) NCI231540, while enzyme is seen in element colors.

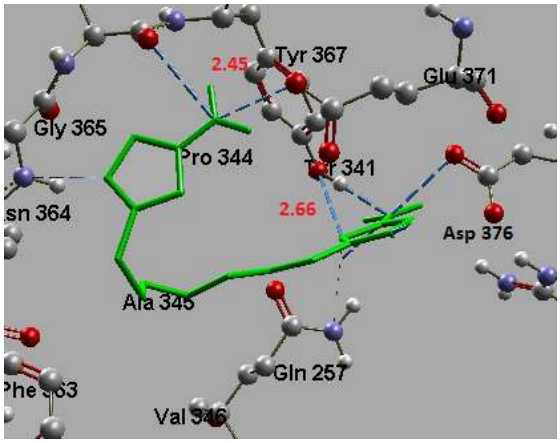


Figure 3: Docking of inhibitor 3 (NCI0382943) into iNOS(PDB code: 1QW4) shown in green thick stick model. iNOS is represented in ball and stick model in element colors. Hydrogen bond interactions are represented in blue dashed lines with the distance given in red color.

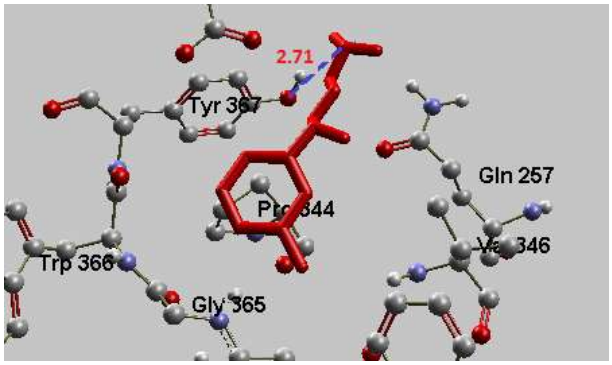


Figure 4: Docking of inhibitor 2 (NCI0231540) into iNOS(PDB code: 1QW4) shown in red thick stick model. iNOS is represented in ball and stick model in element colors. Hydrogen bond interactions are represented in blue dashed lines with the distance given in red color.

REFERENCES

1. Weinberg JB et al. Nitric oxide synthase and cyclooxygenase interactions in cartilage and meniscus: relationships to joint physiology, arthritis, and tissue repair**.** Subcell Biochem 2007, 42:31-62.
2. Murad F. Nitric oxide and cyclic GMP in cell signaling and drug development. N Engl J Med 2006, 355:2003-2011.
3. Stamler JS et al. Nitrosylation. the prototypic redox-based signaling mechanism. Cell 2001, 106:675-683.
4. Cernanec J et al. Influence of hypoxia and reoxygenation on cytokineinduced production of proinflammatory mediators in articular cartilage.Arthritis&Rheumatism. 2002 Apr, 46(4):968-75.
5. Pilla AA**.** Electromagnetic fields instantaneously modulate nitric oxide signaling in challenged biological systems. Biochem Biophys Res Commun. 2012,Sep 28;426(3):330-3.
6. Ghadeer, A.R.Y et al. Pharmacophore and QSAR Modeling of Endothelial Nitric Oxide Synthase Inhibitors and Subsequent Validation and *In Silico* Search for New Hits. Jordan Journal of Pharmaceutical Sciences 2012, 5 (3)**,** 220-241.
7. Hochberg MC et al. The combination of osteoarthritis to disability preliminary data from the women's. Health and Aging Study. J.Rheumatol. 1995; 22 suppl. 43: 16-28.
8. Mireille Bentz et al. Inhibition of inducible nitric oxide synthase prevents lipid peroxidation in osteoarthritic chondrocytes**.** Journal of Cellular Biochemistry 2012,Volume 113, Issue 7, 2256–2267.
9. Stefanovic et al. N-monomethyl arginine an inhibitor of nitric oxide synthase, the development of adjuvant arthritis in rates. Arth Rheum 1994; 37:1062-69.
10. Mazzetti I et al. Differential roles of nitric oxide and oxygen radicals in chondrocytes affected by osteoarthritis and rheumatoid arthritis. Clin Sci 2001; 101: 593–599.
11. Thomsen, R and Christensen, M.H. Mol Dock: A new technique for high-accuracy molecular docking. J. Med. Chem. 2006,49*;* 3315-3321.
12. Wang, X.et al .The PDB bind database: collection of binding affinities for protein-ligand complexes with known three-dimensional structures. Journal of Medicinal Chemistry 2004, vol. 47, no. 12, pp. 2977–2980.