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Research Article

Differential Selectivity of Rat's nNOS-Selective Inhibitors in Human

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Abstract: Nitric oxide synthases (NOSs) consist of three closely related isoforms that produce nitric oxide (NO) a free radical. The overproduction of NO by neuronal nitric oxide synthase (nNOS) is related with a number of neurodegenerative disorders; therefore, selective inhibition of nNOS over eNOS and iNOS is a desirable therapeutic goal. A number of selective inhibitors are reported for nNOS expressed in Rat. We performed a study to test the hypothesis that the inhibitor shows higher selectivity in Rat's nNOS has differential selectivity for human nNOS. Three highly selective rat nNOS inhibitors re-docked with bovine eNOS, mouse iNOS and rat nNOS for validation and with human NOSs for evaluation of the hypothesis. 7- nitroindazole showed selectivity for human eNOS and N-Omega-Propyl-L-Arginine as well as 3,5-Bis(2-(6-amino-4-methylpyridin-2-yl) ethyl) benzonitrile exhibited selectivity for human iNOS. Results suggest that these inhibitors lose their selectivity for human nNOS as displayed for the rat.

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INTRODUCTION

The nitric oxide synthase (NOS) family of enzymes produce nitric oxide (NO) an intracellular signaling molecule that plays an important role in the regulation of many biological processes [1]. In mammalian cells, there are three distinct NOS isoforms: macrophage or inducible NOS (iNOS), neuronal NOS (nNOS) and endothelial NOS (eNOS). These three isoforms share significant amino acid sequence identity (~50-55%) and catalyze the oxidation of L-arginine to citrulline and NO with NADPH and O2 as co-substrates [2]. The NOS C-terminal domain or reductase domain bears remarkable sequence similarity to the mammalian cytochrome P450 reductase and consisting of NADPH, FAD, and FMN binding sites [3]. The N-terminal domain or oxygenase domain has significant sequence similarity only to the three members of the NOS family and contains a heme prosthetic group as well as cofactor site for tetrahydrobiopterin (H4B) [4]. For catalysis, electrons are donated to the reductase domain from NADPH, which proceeds via FAD and FMN to the oxygenase domain. The heme active site and a cofactor site for tetrahydrobiopterin (H4B) interact with electrons in order to catalyze the conversion of L- arginine to L-citrulline and NO [5].

NO overproduction by nNOS has been related with chronic neurodegenerative pathologies including amyotrophic lateral sclerosis [6], Alzheimer [7] and Parkinson [8] diseases as well as neuronal damage resulting from stroke [9], cerebral palsy [10], and migraine headaches [11]. The decline in pathophysiologic levels of NO by selective inhibition of nNOS has the potential to be useful as an approach to developing new therapeutics for neurological diseases. Because each one of the three NOS isozymes is associated with different vital functions: iNOS is devoted to the immune response [12], nNOS to neuronal signaling [13] and eNOS to blood pressure regulation and smooth muscle relaxation [14]. Therefore, selective inhibition of nNOS over the eNOS and iNOS is highly essential for the treatment of neurological diseases to avoid detrimental effects related to iNOS and eNOS inhibition [15]. However, NOS isoforms share a similar domain architecture that poses an obstacle to finding a specific inhibitor of a particular isoform [4].

Up to this time, several categories of selective inhibitors of nNOS have been developed and designed for the prevention and treatment of central nervous system (CNS) disorders [16-17]. Moore et al. reported that 7-nitroindazole selectively inhibits nNOS and exhibits antinociceptive activity in the mouse without increasing blood pressure [18]. In 1997, Zhang et al. found that N-Omega-Propyl-L-Arginine to be a competitive inhibitor of all three isoforms with the potency of nNOS (bovine) inhibition 3158 times that of iNOS (mouse) and 149-fold that of eNOS (bovine) [19]. Recently, Huang et al. reported their best inhibitor 3, 5-Bis (2-(6-amino-4-methylpyridin-2- yl) ethyl) benzonitrile exhibited low nanomolar inhibitory potency and excellent nNOS (rat) selectivity over eNOS (bovine) and iNOS (murine macrophage) were 472-fold and 239-fold, respectively [20]. Although these inhibitors showed great potency and excellent selectivity, they still suffered from serious limitations.

HYPOTHESIS

Studies suggest that the mammalian NOSs share a similar domain architecture [4], although there are some structural differences, which can affect the selectivity of inhibitors in the human. Therefore, present in silico study designed to test the hypothesis that the inhibitor shows higher selectivity in Rat's nNOS has differential selectivity for human nNOS.

EXPERIMENTAL VALIDATION OF THE HYPOTHESIS

Retrieval and Preparation of structures of NOS

The atomic coordinates for the 3D crystal structure of three NOS isozymes determined by X-Ray Diffraction method were downloaded from RCSB-protein data bank (Table 1). These structures were prepared for the docking with the help of UCSF Chimera [26]. The structures 4CX7, 4D1N, 4D1O, 4UX6, 1RS8, and 5AD6 have in total 4, 4, 2, 2, 2 and 2 chains respectively. These are represented by one sequence-unique entity. Chain B of all structures was saved in .pdb files for further refinement and study. By keeping H4B and Heme in the structure, all ligands and water molecules were removed from the Chain B. Further, deletion of solvent and addition of hydrogens were performed. Charges are added to standard residues (AMBERff14SB) and to other residues (AM1-BCC) and are computed using ANTECHAMBER [27]. Subsequently, energy minimization performed for NOSes structures by steepest descent method with 100 steps (step size 0.02A) and conjugate gradient method with 10 steps (step size 0.02A).

Retrieval and preparation of structures of inhibitors

The 3D Conformer of 7-nitroindazole (PubChem CID: 1893) and N-Omega-Propyl-L-Arginine (PubChem CID: 447180) were downloaded from NCBI PubChem database in .sdf format. Whereas, 3D structure of 3,5-Bis (2-(6-amino-4-methylpyridin-2-yl) ethyl) benzonitrile was retrieved from rat nNOS (PDB id 4IMW) structure [20]. The energy of these inhibitors was minimized by applying mmff94 force field and conjugate gradients optimization algorithm for 200 steps with the help of PyRx-Python prescription 0.8 [42] and saved in the .pdb file.

Molecular Docking steps

Auto Dock Tools 1.5.6 [29] performed docking experiments for the NOSs and inhibitors. Prior to docking Kollman charges and Gestgeiger charges were added to NOS structures. After merging nonpolar hydrogen and applying torsions to the inhibitors by rotating all rotatable bonds, Gestgeiger partial charges were assigned. A grid box, that covers the entire binding site of the catalytic oxygenase domain and provides enough space for the inhibitors rotational and translational walk were generated for all three isozymes of the NOS. Docking was performed by keeping the number of points 100, 80, and 110 in X, Y and Z dimension, with 0.375 A° spacing. Whereas values for grid center were kept auto by choosing center on macromolecule for X, Y and Z coordinates. Rigid docking was performed by using Lamarckian genetic algorithm search parameters with 27000 maximum number of generations, 30 independent runs, 150 population size, 2500000 maximum number of energy evaluations [30]. The rates of crossover, the rate of gene mutation, the number of top individuals to survive to next generation (elitism) were defaulting parameters i.e., 0.8, 0.02, and 1 respectively for all 3 isozymes of NOS.

RESULTS AND DISCUSSION

Validation of the study -docking with non-human NOSes

To validate the docking setup accuracy all inhibitors were docked with non-human NOSes catalytic domain as per the previous studies [18-20]. The lowest binding energy, Ki values and isoform selectivity of inhibitors for non-human NOSs are presented in Table 2.

The preliminary study based on binding energy and Ki shows that all inhibitors bind to the catalytic site of nNOS with lower binding energy (or higher affinity) than eNOS and iNOS. Isoform selectivity analysis revealed that all inhibitors have significant e/n and i/n selectivity. 7-nitroindazole and 3,5-Bis(2-(6-amino-4-methylpyridin-2-yl) ethyl) benzonitrile show higher e/n selectivity than i/n. whereas, N-Omega-Propyl-L-Arginine has the higher i/n selectivity than e/n. Moreover, 3,5-Bis(2-(6-amino-4-methylpyridin-2-yl) ethyl) benzonitrile has the higher selectivity for nNOS than other two inhibitors. Further, these inhibitors analyzed for their possible interaction with active site residues of rat nNOS (presented in Table 3).

As discovered by Huang et al., 3,5-Bis(2-(6-amino-4-methylpyridin-2-yl) ethyl) benzonitrile exhibited low nanomolar inhibitory potency and good isoform selectivities. This inhibitor binds to nNOS and eNOS with different binding modes due to the Asp597/Asn368 difference and therefore exhibits the best selectivity. It forms H-bonds with Hem750, Glu592, Asp597 and Trp587 of rat nNOS catalytic domain residues [20]. In present docking study 3,5-Bis (2-(6- amino-4-methylpyridin-2-yl) ethyl) benzonitrile inhibitor bound in the heme active site of rat nNOS with lowest binding energy -9.84 kcal/mol than bovine eNOS (-8.87 kcal/mol) and mouse iNOS (-9.09 kcal/mol). In addition, it forms H-bonds with Hem750, Glu592, Asp597, Trp587 as well as with other Tyr562, Tyr588, Pro565, Asn569, Asp709, Val567, Phe584, Ser477, Gln478, Gln707, H4B760 residues of rat nNOS. Similarly, 7-nitroindazole and N-Omega-Propyl-L- Arginine have the higher selectivity for nNOS over eNOS and iNOS as reported by Moore et al. [18] and Zhang et al. [19], which validated the accuracy of our docking studies.

Table 1: PDB 3D crystal structures used for present study

PDB ID	NOS	Organism	Resolution	Chains	Deposited by
5AD6	nNOS	***Rattus norvegicus***	2.01 A	A, B	Li et al. [21]
1RS8	eNOS	***Bos taurus***	2.3 A	A, B	Flinspach et al. [22]
4UX6	iNOS	***Mus musculus***	3.1 A	A, B	Cheshire et al. [23]
4D1N	nNOS	***Homo sapiens***	2.03 A	A, B, C, D	Li et al [24]
4D1O	eNOS	***Homo sapiens***	1.82 A	A, B	Li et al [24]
4CX7	iNOS	***Homo sapiens***	3.16 A	A, B, C, D	Li et al [25]

Table 2: Docking score and isoform selectivity of inhibitors for active site of non-human NOSes

nNOS selective inhibitors	Bovine eNOS (PDB id: 1RS8)		Mouse iNOS (PDB id: 4UX6)		Rat nNOS (PDB id: 5AD6)		Selectivity	
	LBE	Ki	LBE	Ki	LBE	Ki	e/n	i/n
7-nitroindazole	-6.40	20.52	-7.07	6.62	-7.18	5.48	3.74	1.20
N-Omega-Propyl-L-Arginine	-6.99	7.54	-6.81	10.18	-7.06	6.67	1.13	1.53
3,5-Bis (2-(6-amino-4-methylpyridin-2-yl) ethyl) benzonitrile	-8.87	0.31433	-9.09	0.21806	-9.84	0.06139	5.12	3.55

LBE- Lowest Binding Energy in kcal/mol; Ki- Estimated Inhibition Constant in pM; Ki calculated from binding energy (kcal/mol) by Autodock 4; e/n and i/n are the selectivity ratios of Ki (eNOS or iNOS) to Ki (nNOS)

Table 3: Binding interaction of inhibitors with active site of rat nNOS

Inhibitors of nNOS	nNOS catalytic domain residues involved in H-bonding (30 docking runs)
7-nitroindazole	Val567, Hem750
N-Omega-Propyl-L-Arginine	Glu592, Trp587, Asn569, Gln478, Asp597, Hem750, Arg603, Tyr588, Ser477, Asp709, Gln707
3,5-Bis (2-(6-amino-4-methylpyridin-2-yl) ethyl) benzonitrile	Glu592, Asp597, Hem750, Tyr562, Tyr588, Pro565, Trp587, Asn569, Asp709, Val567, Phe584, Ser477, Gln478, Gln707, H4B760

Table 4: Docking score and isoform selectivity of inhibitors for active site of human NOSes

nNOS selective inhibitors	eNOS (PDB id: 4D1O)		iNOS (PDB id: 4CX7)		nNOS (PDB id: 4D1N)		Selectivity	
	LBE	Ki	LBE	Ki	LBE	Ki	e/n	i/n
7-nitroindazole	**-7.10**	6.30	-6.64	13.62	-6.85	9.51	0.66	1.43
N-Omega-Propyl-L-Arginine	-6.95	8.07 uM	**-9.11**	0.20869	-7.40	3.78	2.13	0.06
3,5-Bis (2-(6-amino-4-methylpyridin-2-yl) ethyl) benzonitrile	-8.49	0.60123	**-10.51**	0.01980	-9.36	0.13768	4.37	0.14

LBE- Lowest Binding Energy in kcal/mol; Ki- Estimated Inhibition Constant in pM; Ki calculated from binding energy (kcal/mol) by Autodock 4; e/n and i/n are the selectivity ratios of Ki (eNOS or iNOS) to Ki (nNOS)

Evaluation of the hypothesis-docking with human NOSes

For evaluation of the hypothesis, docking was performed to predict the possible binding conformation of inhibitors with human NOSes catalytic domain. Table 4 summarizes the lowest binding energy, Ki values and isoform selectivity of inhibitors for human NOSes.

Docking study with human NOSes revealed that 7-nitroindazole has the higher affinity for eNOS whereas N-Omega-Propyl-L-Arginine and 3,5-Bis(2-(6-amino-4-methylpyridin-2-yl) ethyl) benzonitrile have the higher affinity for iNOS. 7-nitroindazole shows significant i/n selectivity ratio (more than one) but exhibits higher eNOS selectivity over nNOS and iNOS. However, N- Omega-Propyl-L-Arginine and 3,5-Bis(2-(6-amino-4-methylpyridin-2-yl) ethyl) benzonitrile have nNOS selectivity only over eNOS but exhibit higher selectivity for iNOS over both nNOS and eNOS. Therefore, we can say that these inhibitors lose their selectivity for nNOS for human as displayed for the rat.

CONCLUSION

Three highly selective rat nNOS inhibitors namely 7-nitroindazole, N-Omega-Propyl-L-Arginine and 3,5-Bis (2-(6-amino-4-methylpyridin-2-yl) ethyl) benzonitrile were screened in silico against the three isozymes of human NOSes for their selectivity. These inhibitors displayed nNOS selectivity when docked with non-human NOSes but lose their selectivity when docked with human NOSes. 7-nitroindazole shows selectivity for eNOS instead of nNOS. Similarly, N- Omega-Propyl-L-Arginine and 3,5-Bis(2-(6-amino-4-methylpyridin-2-yl) ethyl) benzonitrile show selectivity for iNOS. The conformational differences of human and non-human NOS and inhibitors chemical properties may be responsible for their differential selectivity. Therefore, we recommend that prior to

proceeding with the clinical trial of a selective inhibitor, in vivo or in vitro evaluation should be performed to check their human nNOS selectivity.

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CONFLICT OF INTEREST

Author declare that no competing interests exist.

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