http://mnj.ub.ac.id/
DOI: 10.21776/ub.mnj.2023.009.02.12
eISSN: 2442-5001 **pISSN:** 2407-6724
Accredited by DIKTI Decree No: 21/E/KPT/2018

MALANG NEUROLOGY JOURNAL MNJ

RESEARCH ARTICLE OPEN ACCESS

BIOINFORMATICS STUDY OF 7,8-DIHYDROXYFLAVONE AS A NEUROPROTECTIVE AGENT IN ISCHEMIC STROKE VIA TRKB REGULATION AND GLUTAMINASE INHIBITION

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Article History:
Received: July 21, 2022
Accepted: April 27, 2023
Published: July 1, 2023

Cite this as:
Muhammad RF, Adnani B, Arviana SD, Violita AH, Khotimah H, Kurniawan SN, Syaban MFR, Wadjib YYP, Rahayu M. Bioinformatics study of 7,8-dihydroxyflavone as a neuroprotective agent in ischemic stroke via TrkB regulation and glutaminase inhibition. Malang Neurology Journal; 2023.9:144-150. DOI: http://dx.doi.org/10.21776/ub.mnj.2023.009.02.12

ABSTRACT

Background: Stroke, particularly ischemic stroke, is one of the leading causes of death worldwide. Ischemic stroke causes a failure of oxidative phosphorylation and ATP synthesis, resulting in high levels of reactive oxygen species (ROS), neuroinflammatory responses, and apoptosis, all of which result in cell death. Neuroprotective agents are given to prevent the infarct area from expanding.
Objective: This study aims to predict an in silico interaction by 7,8-dihydroxyflavone as neuprotective agent through TrkB signaling and inhibiting Glutaminase activity.
Methods: In silico simulation with 7,8-dihydroxyflavone (DHF) as neuroprotective agent using PubChem, RCSB, Biovia Discovery Studio, PyRx, and PyMol software. This study analyzes the pharmacokinetics, pharmacodynamics, and protein-ligand interactions between 7,8-DHF as a ligand with TrkB (4AT5) and Glutaminase (5JYO) as protein target, compared to their native ligand.
Results: 7,8 DHF binds to 4AT5 and 5JYO with lower bond energy (-9.4 Kcal/mol and -6.3 Kcal/mol, respectively) than the native ligand (-5 Kcal/mol and -5.9 Kcal/mol, respectively). It means that 7,8-DHF may increase protective mechanism.
Conclusion: These findings tend to increase downstream signaling pathways, leading to increased TrkB expression, which induces protective mechanisms, and decreased glutamate expression, which reduces glutamate toxicity.

Keywords: 7,8-dihydroxyflavone, in silico, ischemic stroke, TrkB, glutaminase, glutamate

Introduction

Stroke is the world's second largest cause of mortality and can result in major long-term disability. Every year, there are 13.7 million new cases of stroke, with 5.5 million deaths.[1,2] This condition is characterized as rapidly developing clinical signs of a localized or global impairment of brain function that lasts more than 24 hours or terminates in death and has no other obvious cause than vascular disease.[3,4] Ischemia is the most prevalent cause of stroke, accounting for 87% of all cases.[5] Blockage of oxygen to the brain due to ischemic stroke can lead to failure of oxidative phosphorylation and ATP synthesis. This resulted in excitotoxicity of glutamate which is the first molecular mechanisms in the brain tissue damage, where the release and inhibition of reuptake excitatory amino acid glutamate is rapid and massive.[6,7] Excitotoxicity is influenced by the enzyme glutaminase which catalyzes the hydrolysis of glutamine to produce glutamate in the central nervous system.[8] This causes high levels of reactive oxygen species (ROS), neuroinflammation, and apoptosis, all of which can lead to cell death.[9]

Intravenous thrombolysis (IVT) with recombinant tissue-type plasminogen activator (rtPA) and endovascular therapy (EVT) with a stentriver are currently commonly utilized in the treatment of ischemic stroke. Although this therapy is effective in opening blocked cerebral blood vessels in some patients and can give good results,[10] because this therapy cannot be done in patients with earlier symptom onset of more than 4.5 hours. In addition, on EVT therapy, only 7-13% of patients meet the criteria. Both of these therapies are known to have side effects such as intracranial hemorrhage.[11] Additionally, neuroprotective agents can be used to keep the infarct area from spreading.[12,13] On the other hand, cells can respond to brain damage due to ischemic stroke through the activation of endogenous neurotrophins that can reduce cell damage due to obstruction of blood flow, but their numbers are unable to compensate for the damage caused by ischemic stroke.[14] Neurotrophin is an essential protein released for

growth, development, survival and recovery of the nervous system. One type of neurotropic is BDNF, which can bind to high affinity receptors such as TrkB.[15]

TrkB signaling plays an important role in synaptic maturation, synaptic plasticity, neurite development and arborization, as well as maintaining normal cognitive function.[15] BDNF/TrkB binding then activates the phosphatidylinositol 3-kinase (PI3K) signaling pathway, mitogen-activated protein kinase MAPK and Phospholipase C-γ (PLC-γ) through autophosphorylation and receptor dimerization.[16,17] These three pathways can then increase Hypoxia-inducible factor-1 alpha (HIF-1α) protein synthesis and inhibits prolyl hydroxylase enzyme activity, which can stabilize HIF-1 and activate the HIF-1α transcriptional response to further promote cell survival, anaerobic metabolism, and angiogenesis, including endothelial nitric oxide (eNOS), erythropoietin (EPO), glucose transporter 1 (GLUT1), heme oxygenase 1 (HO-1), and vascular endothelial growth factor (VEGF).[18,19] 7,8-Dihydroxyflavone (7,8-DHF) is a flavonoid that has neurotrophic effects in various neurological diseases such as stroke. With its ability to cross the blood-brain barrier, this isolate can mimic BDNF activity through its role as a specific TrkB receptor agonist.[20]

The research to observe drug design needs a long time, but screening to find out potential compounds as drug candidates at this time, the in silico approach can be used.[21,22,23] In silico is a research approach that uses computer simulation and available database information.[24] This computation method can predict the interaction between ligand and target protein, as well as its bond energy.[25] This study aims to determine the potential of 7,8-DHF as an ischemic stroke therapy through the molecular interaction with TrkB and Glutaminase enzyme.

Methods

Ligand and Protein Preparation

The ligand used in this study was 7.8-dihydroxyflavone (DHF), which was downloaded as a.sdf file from PubChem (http://pubchem.ncbi.nlm.nih.gov/). The proteins Tyrosine Kinase B (PDB ID: 4AT5) and Glutaminase (PDB ID: 5JYO) came from the RSCB website (https://www.rcsb.org/search).[26] The ligand minimization process was carried out using OpenBabel in PyRx software, which allowed the ligands to be more flexible, and then the file structure data format (.sdf) was changed to protein databank format (pdb).[27] Using BIOVIA Discovery Studio, protein stabilization was performed to adjust to the body's physiology by removing water and hydrogen atoms.[28] The control ligands were downloaded as .sdf. files from RSCB. cFMS Kinase Inhibitor was used as a control ligand for Tyrosine Kinase B, and Telaglenastat was used as a control ligand for Glutaminase.

Drug-likeness and Biological Activity Prediction

The pharmacokinetics of the 7,8-DHF ligand molecule was examined as a drug candidate using the SwissADME webserver (http://swissadme.ch), followed by a drug-likeness analysis utilizing the Lipinski Rule of Five website (scfbio-iitd.res.in).[29,30,31] Furthermore, the bioactivity of the ligands as a neuroprotector agent is screened using Way2Drug Prediction of Activity Spectra for Substance (http://www.pharmaexpert.ru/passonline/) to determine the quantitative structure-activity relationship.[32] A protein kinase stimulant is the expected bioactivity.

Molecular Docking

The binding energy value created when a ligand binds with its receptor is determined using molecular docking.[28,33] In this study, specific docking refers to comparing the binding energies of 7,8-DHF and a control ligand that binds to the same binding site. The PyRx application Vina Wizard is used in this study to perform molecular docking simulations. The PDBQT file format was used to evaluate the receptor and ligand files.[27,34]

Protein-ligand Interaction Analysis

Docking data were displayed at the molecular level using the PyMol and BIOVIA Discovery Studio tools. A protein-ligand bond analysis was carried out based on the interaction and type of bond established by the 7.8-DHF when it binds to target proteins.[35,36] The Discovery Studio application was used to investigate the ligand-protein interactions. After docking using the preceding VinaWizard, the program will generate a representative 2D schematic representation of the complex interaction between the ligand and the receptor.[37,38]

Results

Drug-likeness and The Biological Activity Prediction of 7,8-dihydroxyflavone

The pharmacodynamic tests in this study were carried out by uploading SMILES of 7,8-DHF compound on the PASS online site (http://way2drug.com/PassOnline/). The analysis results showed that the probable activity (pa) value of 7.8-DHF in stimulating protein tyrosine kinase was 0.187, higher than the value of probable inactivity (pi). This finding suggests that 7.8-DHF is effective in tyrosin kinase protein stimulation (pa>pi) (Table 1).

Table 1. Pharmcodynamic Test of 7,8-DHF

Compound	Probable activity (Pa)	Probable inactivity (Pi)	Action
7,8-DHF	0,187	0,045	Tyrosine kinase stimulant

The pharmacokinetic activity of 7,8-DHF was tested using SwissADME (http://swissadme.ch/), and showed the human intestine absorption (HIA) of 7,8-DHF, the ability to penetrate blood brain barrier (BBB), and the protein plasma binding that displayed on Table 2.

Table 2. Pharmacokinetic Test of 7,8-DHF

HIA	BBB	Plasma Protein Binding
92.63%	0.93	93.02%

The Lipinski Rule of Five is used to determine the level of compound similarity that has a certain biological activity which can then be determined the compund feasibility as a candidate for a new drug. This test is carried out on the Lipinski test site (http://www.scfbio-iitd.res.in/software/drugdesign/lipinski.jsp). The result showed in Table 3.

Table 3. Lipinski Rule of Five Test Results of 7,8-dihydroxyflavone

Compound	Molecular weight	Hydrogen bond donor	Acceptor bond donor	LogP	Molecular Refractility	Lipinski Rule
7,8-DHF	254 g/mol	2	4	2.71	69.15	Yes

Table 4. Binding Affinity Result

Protein Target	RSCB ID	Binding Affinity (Kcal/mol)		
		Native Ligand		7,8-DHF
TrkB	4AT5	cFMS Kinase Inhibitor	-5	-9.4
Glutaminase	5JYO	Telaglenastat	-5.9	-6.3

Table 5. Active site, dimension, and docking center grid

Protein Target	Native Ligand	Amino Acid Residue	Dimension	Docking Center Grid
4AT5	cFMS Kinase Inhibitor	Tyr635; Asp710; Phe633 Leu699; Leu560; Ala586 Val568; Leu608; Val617 Lys588; Unk1	X :10 Å Y : 10 Å Z : 10 Å	X : -13.01 Y : 28.07 Z : -17.54
	7,8-DHF	Leu608; Val617; Phe633 Leu699; Asn697; Phe711 Asp710		
5JYO	Telaglenastat	Lys320; Leu321; Leu323; Tyr394; Ala390	X :17 Å Y : 17 Å Z : 17 Å	X : -5.48 Y : -71.02 Z : 16.47
	7,8-DHF	Leu321; Phe322; Asp327; Tyr394		

Molecular Docking

The molecular docking test in this study was carried out using the Vina Wizard subprogram on the PyRx software specifically. This test was conducted to identify the molecular interaction between 7,8-DHF ligand on TrkB receptor proteins, then the binding affinity value was compared with the native ligand for each protein. The binding affinity results can be seen in Table 4.

Protein-ligand Interaction Analysis

The amino acid residue can be found from the bond analysis between protein receptor and the ligand. Molecular docking can be used to determine the type and bond strength, and compare the bonds with each other. The protein active site and docking grid center can be seen in Table 5.

Discussion

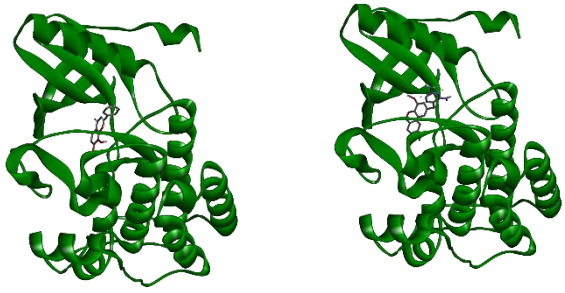
The results of 7.8-DHF pharmacodynamic tests carried out on the PASS online site showed that the compound was good in stimulating protein tyrosine kinase because the value of probable activity (pa) higher probable inactivity (pi), but had a low experimental value because has a pa value less than 0.5 (Table 1). The low pa value could be due to the fact that 7,8-DHF is a new compound with low biological activity,[32] as well as research on the effect of 7,8-DHF as a neuroprotectant. Pharmacokinetic analysis in this study used the PreADMET Online website to analyze the absorption of 7,8-DHF in the human intestine, the ability to penetrate the blood brain barrier (BBB), and its binding to plasma proteins.

The HIA value of 7.8-DHF was 92.63% (Table 2), including the high category (70-100%) which indicates that this compound can be well absorbed by the intestine. Wang et al. (2017) stated that the relationship between oral bioavailability and intestinal absorption has been proven by many previous studies so that it can be concluded that the majority of compounds (64%) are mainly controlled by the intestinal absorption process.[39] When this compound has a high intestinal absorption rate, 7,8-DHF can be well absorbed and diffuse passively into the blood vessels which will then enter the systemic circulation until it reaches the site of action to give the desired effect.[40]

The ability of 7,8-DHF to penetrate BBB is in moderate category because the value was 0.93 (Table 2). The value >2.0 is high category, while 0.1-2.0 is medium category, and <0.1 is low category.[41] Compounds that can pass through BBB are only compounds that dissolve well in fat, so 7.8 DHF is a fat-soluble. It is known that 7,8-DHF can cross the BBB through a diffusion mechanism.[40] This study also showed the 7,8-DHF ability to bind plasma proteins had strong category (> 90%) (Table 2) so that when this compound decreases plasma distribution, the presence of strong plasma protein binding can replace the reduced plasma concentration because 7,8-DHF also has a high rate of intestinal absorption.[41] The high binding of 7,8-DHF with plasma proteins also indicates that 7,8-DHF can be protected from oxidation, minimize toxicity, and increase the half-life of these compounds. These results also indicate that the binding of 7,8-DHF and plasma proteins is lipophilic so that 7,8-DHF remains in the blood vessels to reach the site of action and does not diffuse to other places.[40] According to the drug-likeness prediction, 7,8-DHF met all The Lipinski Rule criteria with the molecular weight <500 g/mol, hydrogen bond donor <5, hydrogen bond acceptor <10, lipophilicity (LogP) <5, and molar refractivity between 40-130 (Table 3). This test is carried out to evaluate the similarity of a drug and determine whether a compound with pharmacological or biological activity can become orally active in humans.[42] A compound is categorized as eligible as a drug candidate if it meets at least 2 of the 5 criteria.[43]

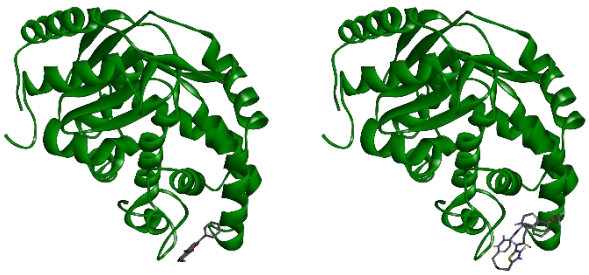
The molecular docking outcomes visualization can be seen on Figure 1, showed that protein-ligand interaction with the lowest energy bond can affect protein's biological activity. Based on Table 5, the binding affinity value between 4AT5 and 7.8-DHF is -9.4 Kcal/mol, while the binding affinity for

4AT5 with native ligand is -5 Kcal/mol. These results indicate that 4AT5 bond with 7,8-DHF are more stable than with native ligands. The low binding energy suggests a role for 7,8-DHF in the TrkB stimulation and its downstream signaling pathways that play a role in neuronal development and survival, synaptic plasticity, and anti-apoptotic mechanisms.[44,45] One study found that 7,8-DHF has a neurotrophic effect in monkeys by shielding dopaminergic neurons from apoptosis caused by 1-methyl-4-phenylpyridinium (MPP+)-induced neurotoxicity. The study also found that long-term (7 months) therapy with 7.8-DHF did not produce toxicity in monkeys at a dose of 30 mg/kgBW, which is 5 times greater than in rats (5 mg/kgBW), implying that 7.8-DHF is safe as a possible clinical pharmacological agent.[46] The activation of TrkB receptor also can be induced by administration of 7,8-DHF as much as 5 mg/kgBB/day for 5 weeks and show neuroprotective properties that decreasing the number of degeneration of dopaminergic neuron cells in the subtantia nigra and striatum parts of the brain in Parkinson's Disease model mice. This result is obtained from the activation of cascade signaling that decrease *phospho*-MAPK, *phospho-α*-synuclein and phospho Tau which are typical marker protein in Parkinson's disease.[47]



a. **b.**

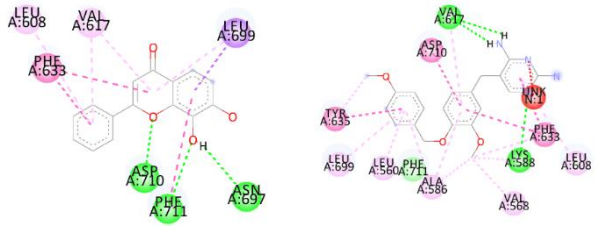
Figure 1. Visualization of 3D molecular interaction between ligand and TrkB (PDB ID: 4AT5). Interaction with (a) 7.8-DHF; (b) Native ligand



a. **b.**

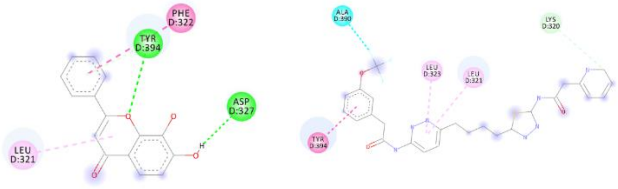
Figure 2. Visualization of 3D molecular interaction between ligand and Glutaminase (PDB ID: 5JYO). Interaction with (a) 7.8-DHF; (b) Native ligand

A study regarding the effect of 7,8-DHF in protecting retina after retinal ischemic-reperfusion injury (RIRI) showed that levels of NF-kB and phosphorylated NF-kB (pNF-kB) were significantly increased in the RIRI condition and recovered by 7.8-DHF. In addition, some of the RIRI-induced increases in inflammatory factors such as IL-1β, IL-6, TNF-, and IFN-γ also can be reversed after 7,8-DHF therapy.[48] The study also showed that the underlying signaling such as pAkt/Akt and pErk/Erk is inhibited by RIRI and can be recovered by 7,8-DHF. Apoptotic-related factors such as Caspase 3 and Bax were also elevated in RIRI but were reversed by 7,8-DHF. Furthermore, 7,8-DHF compounds are able to inhibit the activity of prolyl hydroxylase enzymes via the PI3K/AKT signaling pathway to activate the HIF-1α gene and its underlying signaling pathways to further activate the mechanism of adaptation to hypoxia and minimize the volume of post-ischemia infarction.[49] Akt phosphorylation causes activation of Bcl-2, FoxO3a, mTOR, and glycogen synthase kinase-3 to inhibit the occurrence of apoptosis.[50] Based on these study, it can be concluded that 7,8-DHF is able to activate the BDNF/TrkB signaling pathway and reduce the rate of apoptosis.[45]



a. **b.**

Figure 3. Visualization of the bond type formed between ligand and TrkB (PDB ID: 4AT5). Interaction with (a) 7.8-DHF; (b) Native ligand



a. **b.**

Figure 4. Visualization of the bond type formed between ligand and Glutaminase (PDB ID: 5JYO). Interaction with (a) 7.8-DHF; (b) Native ligand

Figure 2 shows the same results when comparing 7,8-DHF to its native ligand in the interaction with Glutamine. 5JYO and 7.8-DHF have a binding affinity of -6.3 Kcal/mol, whereas 4AT5 with its native ligand has a binding affinity of -5.9 Kcal/mol, as shown in Table 5. This shows that 7,8-DHF has a higher level of stability than its native ligand. This demonstrates that 7,8-DHF is more stable than its natural counterpart. The role of 7,8-DHF is to inhibit glutaminase, a catalytic enzyme that catalyzes the hydrolysis of glutamine to glutamate and is thought to be involved in the formation of excitotoxic glutamate in CNS nervous disorders.[51] As previously stated, the glutamate transporter's activity stops in pathological conditions such as ischemia/reperfusion injury or aging, and glutamate that is ubiquitous in the extracellular can damage the surrounding nervous tissue and cause brain cell death due to excitotoxicity.[52] Glutamate can binds to glutamate receptors and causes Ca^{2+} to enter the cell lumen. The continued increase in intracellular Ca^{2+} will lead to pathological conditions in which cells become over-excited, causing the release of harmful substances such as reactive oxygen species (ROS), ATPase, endonuclease, proteases, and phospholipases, which are Ca^{2+}-dependent degradative enzymes. The release of this enzyme will disrupt the cell

membrane, allowing more and more harmful chemicals to enter the cell, as well as the release of apoptotic signals from the mitochondria, which will activate caspase by caspase, resulting in cell death. Peri-infarction depolarization is the process by which a harmful signal can spread from a dying cell to nearby healthy cells. Ischemic spread can occur, resulting in decreased perfusion and secondary expansion of the infarcted core, potentially involving the penumbral area[53]. Another thing to consider is that controlling glutamate release is notoriously difficult. This is due to the fact that glutamate release is controlled by six different mechanisms: (I) reversal uptake of glutamate transporters in the plasma membrane, (ii) opening of anion channels due to cell swelling, (iii) Ca^{2+}-dependent exocytosis, and (iv) glutamate turnover via the cysteine-receptor antiporter glutamate, (v) purinergic ionotropic receptor release, and (vi) functional unpaired 'hemichannel' connexons on the cell surface[54]. Our in-silico testing has shown that 7,8-DHF inhibits the glutaminase enzyme, which is a key component in glutamate synthesis. As a result, it can be concluded that using 7,8-DHF reduces glutamate activity by inhibiting glutamate production. There have been no studies to our knowledge that have looked at the effect of 7,8-DHF on the glutaminase enzyme directly, so we hope that these findings will serve as a starting point for future research.

Figure 3 and Figure 4 showed that 7,8-DHF bound to the binding domain of the target protein and showing the type of chemical interaction. The Pi-Pi T-shaped bond residue generally occurs due to the interaction between the phenyl ring on the benzene element of the ligand and target protein.[55] The Pi-Pi T-Shaped type including to hydrophobic bond, where this bond is the main contributor to protein stability so that the hydrophobic bond is the main determinant of the equilibrium folds configuration in various proteins.[56] The Pi-Alkyl bonds also play a role in the stability of the bond structure. Interaction between Pi-Alkyl and Pi-Sigma can also help stabilize ligand and receptor bonds and is known to be able to normalize the dipole moment when energy transfer occurs with surrounding amino acids.[57] Pi-Alkyl and Pi-Sigma bonds are also classified as hydrophobic bonds. The hydrogen bonds at the residues can also help protein stability, but are lower than hydrophobic interactions.[56] Hydrogen bonds are included in non-covalent bonds, where its amount can determine the compound lipophilicity and the ability to penetrate BBB which then acts at the site of action.[58] Our study also shows that there is a van der waals bond which also help stabilize the protein and its ligands.[59] However, we found an unfavorable bump bond on the native ligand group at the Unk1 residue on the bond between the native ligand and TrkB which could affect the stability of the complex because it causes a repulsive force between the 2 molecules and atoms.[60] Fortunately, we did not find this bump bond in the binding between 7,8-DHF and the target protein.

Conclusion

The 7,8-DHF compound has a neuroprotective effect by enhancing downstream signaling pathways that lead to increased TrkB expression, which triggers protective mechanisms, and inhibiting glutaminase enzyme, which reduces glutamate expression and toxicity.

Acknowledgement

The authors thank all the Biomedical Laboratory staff of Brawijaya University for their excellent assistance during this study.

Conflict of Interest

The authors declare that there is no conflict of interest.

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