International Journal of Engineering Research and Technology.
ISSN 0974-3154 Volume 11, Number 1 (2018), pp. 1-10

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Identification of Quercetin as a Potential Anti-Neuroinflammatory Agent using BV2 Microglia Cell System and in Silico Molecular Docking Technology

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Abstract

The purpose of this study was to analyze the anti-neuroinflammatory effect of quercetin, a well known natural flavonoid, to the iNOS(inducible nitric oxide synthase)-mediated nitric oxide production in BV2 microglia and to find its biochemical interacting mechanism on iNOS protein using computational docking technology. For this, quercetin, a major active ingredient of the plum and onion, were used as ligand for molecular interaction. The 3D crystallographic structure of molecular target iNOS was obtained from PDB database (PDB ID: 1M9T). Tetrahydrobiopterin, a iNOS protein ligand was taken as the standard for comparative docking analysis. Quercetin showed maximum binding affinity with a molecular target iNOS with the binding energy of −8.80 kcal/mol as compared to the tetrahydrobiopterin (−7.00 kcal/mol). In the cell biological assay study, quercetin significantly reduced iNOS-mediated nitric oxide production in BV2 microglia. These results strongly indicated that quercetin could be one of the potential drug candidate to protect iNOS-mediated neuroinflammation and brain diseases.

Keywords: Autodock, Drug Design, iNOS, Neuroinflammation, Quercetin

INTRODUCTION

Recent pharamcological approach using computer aided drug discovery have become very important resource to identify the potential drug candidates for various kinds of brain diseases[1]. In silico drug screening technology offers the proper advantage of identifying lead compounds from several potentially useful hit compounds. In silico molecular docking technology is a tool in structural molecular biology and structure-based drug discovery and fast methods to do so[2,3]. Besides, the purpose of ligand-protein docking is to predict molecular recognition, binding modes and predicting binding affinity(kcal/mol)[4]. Scientists in the pharmacology field have employed the

new drug development technologies to determine successfully useful potential binding sites and used the experimental results to indentify, improve and develop drugs that fit better into the binding pocket (active site) in the target proteins[3].

Nitric Oxide (NO) is produced from L-arginine in microglia by iNOS enzymes. iNOS is induced by microbial activating products, such as lipopolysaccharide (LPS) and inflammatory cytokines such as interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α) and interferon-γ (INF-γ)[5]. NO production is enhanced in respond to LPS stimuli and mediates the neuroinflammation. Because of the importance of NO derived from iNOS in inflammatory response, there were several research efforts to find a selective iNOS blocker[6,7,8]. Thus, the compounds down-regulating the activity of iNOS are suggested to be potential as anti-neuroinflammatory agents.

Quercetin(Fig. 1b) is a well known natural antioxidant. It is found in citrus fruit(plum), and onions, and various previous studies proved its potential therapeutic qualities and anti-oxidation effects[9]. But, the biochemical molecular interacting mechanism about the anti-neuroinflammtory effect of quercetin on the iNOS protein has not been well studied. Using in silico molecular docking technique and BV2 microglia cell based assay system, we investigated the potential effect of quercetin as a novel regulator for the iNOS-mediated nitric oxide production in microglia. In this study, the structural 3D model of the quercetin on the iNOS protein active site has been performed, which may expedite further development of more natural iNOS-protectors to control activated microglia-induced neuronal diseases.

MATERIALS AND METHODS

Cell culture

The immortalized murine BV-2 cell line of reactive microglia cells[10] were grown and maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum (Gibco, USA), streptomycin, and penicillin as described previously[11]. Under a humidified 5% CO2/95% air atmosphere and at 37°C, cells were split twice a week and plated in 10 cm^2 Petri dishes (Corning, Acton, MA, USA) at a density of 5×10^5 cells for BV-2 cell line. For the experiments, cells were plated on 6-well dishes (2×10^6 cells/well).

iNOS-mediated NO(nitric oxide) production assay

To stimulate TLR-4, BV-2 cells were washed with phosphate-buffered saline (PBS) twice, replenished with a serum-free DMEM (Gibco, USA) and LPS (Sigma, St. Louis, MO) was added to the culture medium. Quercetin (Sigma, St. Louis, MO), were added to cells 10 min before LPS (100 ng/ml) treatment. NO produced by the BV-2 cells was determined by assaying the levels of Nitrite using the Griess reagent (Sigma, St. Louis, MO). The significance level of the quercetin effect were set at $*p \leq 0.05$ versus LPS alone. All data were represented as the means ± SEM (Standard Error of the Means).

Molecular docking analysis

The three-dimensional structure of iNOS protein (PDB ID: 1M9T)(Fig. 1c) was downloaded from the RCSB protein Data Bank. The chemical structure of tetrahydrobiopterin(Pubchem CID=1125)(Fig. 1a) and quercetin(Pubchem CID=5280343)(Fig. 1b) were obtained from PubChem compound database. It was prepared by ChemBioDraw and MOL SDF format of this ligand was converted to PDBQT file using PyRx tool[12] to generate atomic coordinates. For docking analysis, PDB coordinates of the target protein, tetrahydrobiopterin, and quercetin molecule were optimized by Discovery Studio version 4.5 software[13]. These coordinates had minimum energy and stable conformation. The active sites are the coordinates of the ligand in the original target protein grids, and these active binding sites of target protein were analyzed also using the Discovery Studio version 4.5 and NX-QuickPharm program(Neuronex Inc., South Korea)[14]. A computational ligand-target docking approach was used to analyze structural complexes of the iNOS(target) with tetrahydrobiopterin and quercetin(ligand) in order to understand the structural basis of this protein target specificity. Docking was carried out by PyRx, AutoDock Vina, and NX-QuickPharm option based on scoring functions. The energy of interaction of tetrahydrobiopterin and quercetin with the iNOS protein is assigned "grid point." At each step of the simulation, the energy of interaction of ligand and protein was evaluated using atomic affinity potentials computed on a grid. The significance level of the quercetin binding affinity on the iNOS was set at *, $p \leq 0.05$ versus tetrahydrobiopterin. All data were represented as the means ± SEM (Standard Error of the Means).

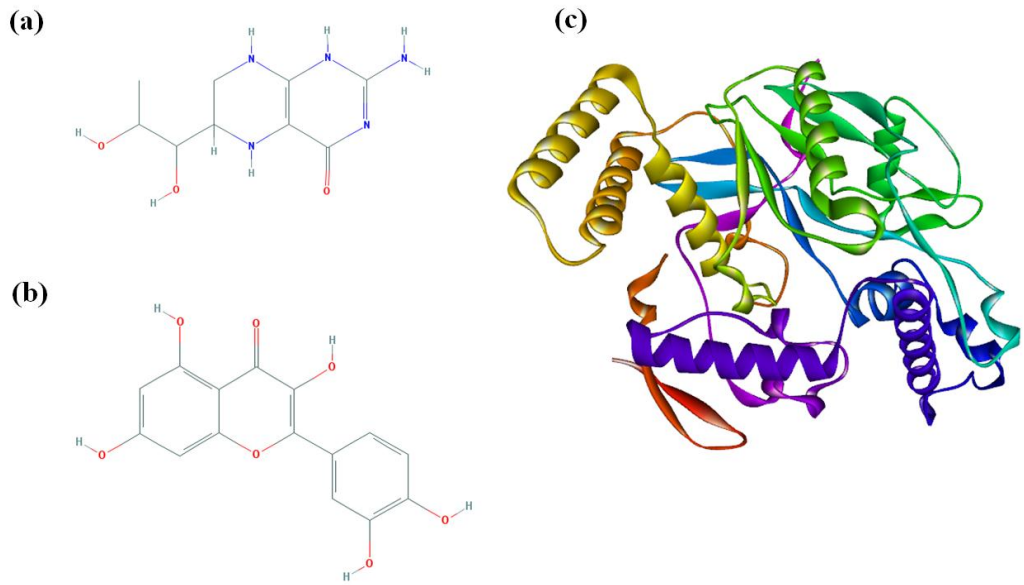

Figure 1. Chemical structure of the (a) tetrahydrobiopterin and (b) quercetin. Three-dimensional (3D) structure of the iNOS protein A domain (PDB ID = 1M9T)

RESULTS

Quercetin binds in high affinity to the iNOS active site

PyRx Autodock 4 docking analysis was applied to investigate the molecular binding interactions of quercetin and tetrahydrobiopterin molecules, respectively with iNOS protein (Fig. 2a) and to elucidate the possible molecular mechanism. As shown in Fig. 2c and Fig. 3b, quercetin interacted with 6 amino acid residues (TRP188, CYS194, GLY196, PHE363, ASN364, GLY365) and tetrahydrobiopterin interacted with 3 amino acid residues (SER112, ARG375, TRP457)(Fig. 2b and Fig. 3a). The average molecular binding affinity (docking energy) scores of the tetrahydrobiopterin and quercetin on the iNOS target were -6.61 kcal/mol (tetrahydrobiopterin, SEM ±0.23) and -8.30 kcal/mol (quercetin, SEM ±0.33) (Table 1).

Table 1. Binding affinity of the tetrahydrobiopterin and quercetin on the target iNOS protein active site.

	Max Binding Affinity (kcal/mol)	Average Binding Affinity (kcal/mol)	SEM (n = # of binding mode)
tetrahydrobiopterin on the iNOS protein	-7.00	-6.61	±0.23 (n=9)
Quercetin on the iNOS protein	-8.80	-8.30*	±0.33 (n=9)

* $p < 0.05$

The numbers of the binding modes of both ligands to the iNOS active site were nine (n=9), respectively. Based on the present molecular docking data, quercetin appeared as a strong binder to the iNOS target protein than the tetrahydrobiopterin.

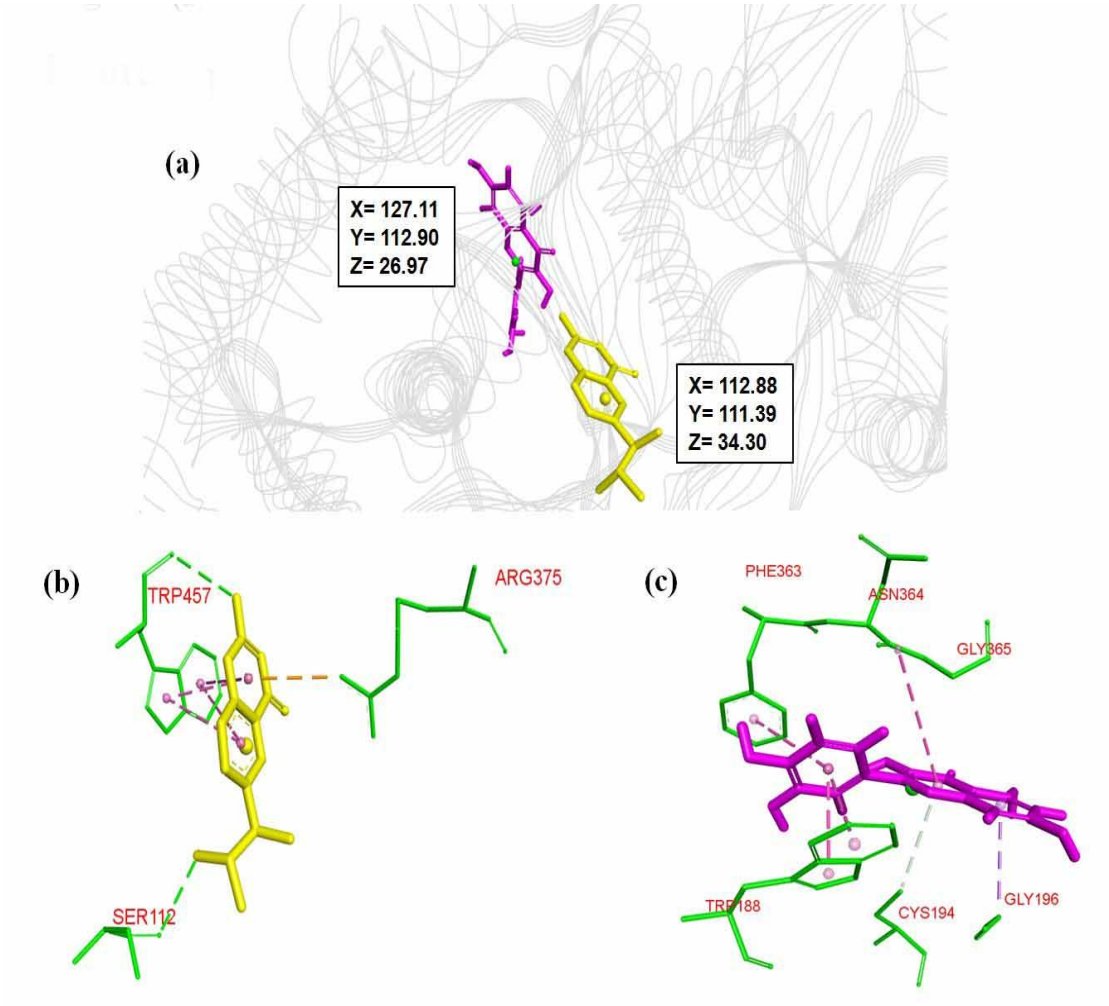

Figure 2. (a) 3D molecular docking pattern and binding position of the tetrahydrobiopterin (yellow color stick) and the quercetin (purple color stick) on the iNOS protein (grey color ribbon) active site. (b) 3D pattern of the interacting amino acids with tetrahydrobiopterin in the iNOS active site. (c) 3D pattern of the interacting amino acids with quercetin in the iNOS active site.

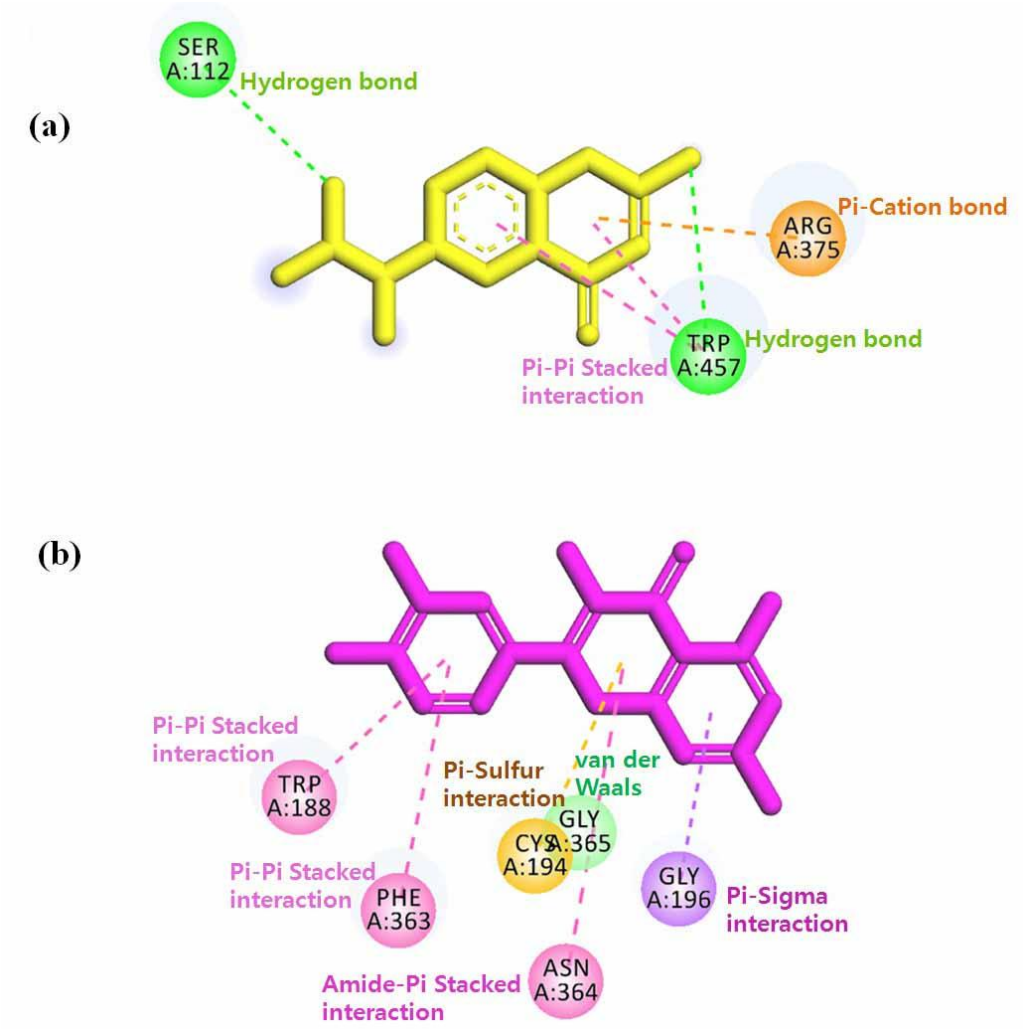

Figure 3. (a) The name of the chemical bonds (or interactions) and 2D pattern of the interacting amino acids with tetrahydrobiopterin in the iNOS active site. (c) The name of the chemical bonds (or interactions) and 2D pattern of the interacting amino acids with quercetin in the iNOS active site.

Effect of quercetin on the iNOS-mediated NO production on the BV2 microglia

The microbial neurotoxin LPS is the well-known target of innate recognition and induces a robust neuroinflammatory response by microglial cells and induces stimulation of the NF-κB signaling pathways and NO synthesis. To elucidate the effect of quercetin on the iNOS-mediated microglial activation, BV-2 cells were incubated with LPS in the absence or presence of quercetin (Fig. 4). Quercetin significantly reduced LPS-induced NO production in a concentration-dependent manner (Fig. 4), indicating that quercetin effectively affects TLR-4 and iNOS cell signaling.

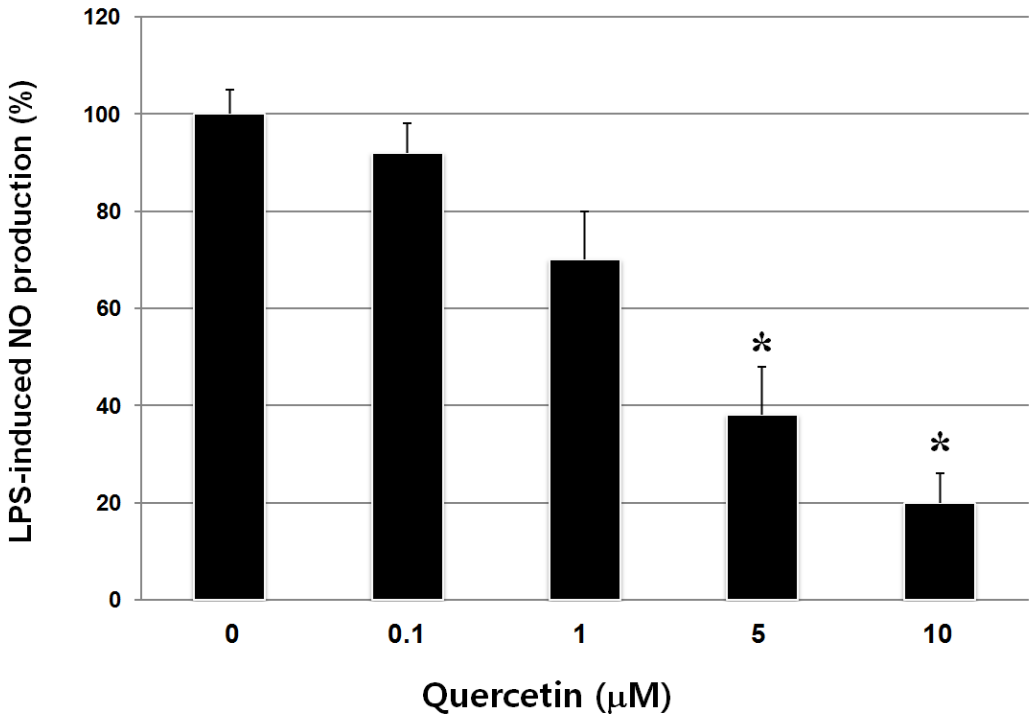

Figure 4. Effects of quercetin on the NO production induced by LPS in BV-2 microglial cells. Data represent means ± SEM of three independent experiments. Significant differences between LPS and quercetin + LPS are presented. *$p < 0.05$.

DISCUSSION

Microglia act as resident brain macrophages that become inflammatory activated in most brain pathologies[15]. In normal state, microglia protect neurons, but may accidentally kill neurons when attempting to limit infections or damage, and this may be more common with degenerative disease as there was no significant selection pressure on the aged brain in the past[15]. Neuroinflammation is one of the main mechanisms involved in the progression of dangerous neurodegenerative diseases, such as Alzheimer, Parkinson, multiple sclerosis, amyotrophic lateral sclerosis and others[16]. Natural herb derived flavonoids, possess neuroprotective potential probably related to their ability to regulate the neuroinflammatory responses involved in neurodegenerative diseases[17,18]. Quercetin(or quercetin enriched herb extracts) can reduce the pro-inflammatory cytokine(COX-2, IL-6, IL-1β, and TNF-α) expressions, down-regulate inflammatory inducers and prevent brain damage[16,19]. The aim of the present study was to evaluate the effect of quercetin, one of the most abundant flavonoids in herbs and fruits, on iNOS-mediated NO production in BV2 microglia. Several previous studies have revealed that anti-neuroinflammatory effects of natural flavonoids in neurodegenerative disorders[16,20,21]. It was noteworthy that quercetin pretreatment significantly protected LPS-TLR4-iNOS-signal pathway

mediated neuroinflammation in microglia cell system(Fig. 4). In accordance with the previous reports[22,23], our data also demonstrated that quercetin effectively protected iNOS-mediated nitric oxide production and neuroinflammation (Fig. 4).

Quercetin has a planner(polyphenol) structure(fig. 1b) favorable to enter into cell membranes effectively and has shown strong affinity with cytoplasmic iNOS protein (Fig 3b and Table 1). The computational docking of iNOS target with quercetin using auto docking procedure revealed that all the lowest energy complexes of iNOS are stabilized by intermolecular aromatic ring-mediated pi-pi stacking interactions and quercetin additionally makes pi-sulfer, amide-pi stacked, pi-sigma interactions than tetrahydrobiopterin does(Fig. 3b). The calculated final docked maximum binding affinity of quercetin to iNOS protein is −8.80 kcal/mol and tetrahydrobiopterin (standard) is −7.00 kcal/mol (Table 2). Therefore, docking results revealed that this quercetin compound can enter the substrate-binding region of the iNOS active site and interacts effectively than tetrahydrobiopterin. The interacting numbers of the chemical bonds and binding patterns of the ligand among the iNOS amino acids might be critical factors for regulating target iNOS protein activity.

CONCLUSION

These results demonstrated clearly that quercetin accurately and effectively interact with iNOS protein target. Therefore, quercetin might play an important role in inhibiting iNOS-mediated nitric oxide production and neuroinflammation. These data also suggest that computer aided drug design process using PyRx, Discovery Studio 4.5, and NX-QuickPharm tools is highly reliable and can be a good example for indentifying the action mechanism between the iNOS and its interacting ligands. In conclusion, quercetin can play an important role in altering the progression of neuroinflammatory diseases by its protective effect against iNOS-mediated oxidative brain stress.

ACKNOWLEDGEMENT

This research was supported by Gimcheon university research fund, (gc-16006).

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