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Investigation of Coumestrol as a Potent IKK-beta Inhibitor Using Microglia Cell System and Computer Aided Drug Design Technology

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Abstract

The purpose of this study was to analyze the anti-neuroinflammatory effect of coumestrol, a well known natural flavonoid, to the IKK-beta protein signaling mediated nitric oxide production in BV2 microglia and to find its biochemical interacting mechanism on IKK-beta protein using computer aided drug designing technology. For this, coumestrol, a major active ingredient of the soybean, were used as a candidate ligand for molecular interaction. The 3D crystallographic structure of molecular target IKK-beta was obtained from PDB database (PDB ID: 4KIK). IKK-2 inhibitor VI ((5-Phenyl-2-ureido)thiophene-3-carboxamide), a well known IKK-beta protein antagonist was taken as the standard for comparative docking analysis. Coumestrol showed maximum binding affinity with a molecular target IKK-beta with the binding energy of −10.40 kcal/mol as compared to the IKK-2 inhibitor VI (−8.00 kcal/mol). In the cell biological assay study, coumestrol significantly reduced IKK-beta-mediated nitric oxide production in BV2 microglia. These results strongly indicated that coumestrol could be one of the potential drug candidate to protect IKK-beta-mediated neuroinflammation and brain diseases.

Keywords: Autodock, Coumestrol, Drug Design, IKK-beta, Neuroinflammation

INTRODUCTION

Coumestrol (Fig. 1b) is a well known natural antioxidant. It is found in alfalfa, clover, and soybean, and various previous studies proved its potential therapeutic qualities and anti-oxidation effects[1]. In addition, a novel activity of coumestrol was reported as it is binding to human ERβ and that represses microglia-mediated inflammation, which is associated with various neurodegenerative diseases, such as multiple sclerosis[2]. But, the biochemical molecular interacting mechanism about the anti-neuroinflammtory effect of coumestrol on the IKK-beta protein has not been well studied.

Computer aided drug design and novel drug discovery have become very important pharmacological resource to identify the potential drug candidates for various kinds of neuroinflammatory diseases[3]. Specially, in silico drug screening technology offers the proper advantage of identifying lead compounds from several potentially useful hit compounds. Thus, computer aided in silico molecular docking technology is a useful tool in structural molecular biology and structure-based drug discovery and fast methods to do so[4,5]. Besides, the final purpose of ligand-protein docking is to predict molecular recognition, binding modes and predicting binding affinity(kcal/mol)[6]. In the pharmacology research field have employed the novel drug development technologies to find out useful potential binding sites and to applicate the experimental results to indentify, improve and develop drugs that fit better into the active site(binding pocket) in the target proteins[5].

Microglial nitric Oxide (NO) is produced and mediated by TLR4 signaling. When TLR4 is stimulated by ligand LPS(Lipopolysaccharide)→MyD88 is recruited via TIRAP-MyD88 recruits IRAK kinases (mainly IRAK4)→IRAKs are phosphorylated→IRAKs dissociate from MyD88→IRAKs interact with the E3 ligase TRAF6→TRAF6 forms a complex with the ubiquitin conjugating enzymes UBE2N/UBC13 and UBE2V1/UEV1A→This complex polyubiquitinates target proteins such as TAK1/TAB1/TAB2/TAB3→Polyubiquitination leads to TAK complex activation and subsequent IKK activation[7]. Next, IkappaBs are destroyed by the 26S proteasome resulting in translocation of NF-kB to the nucleus and regulation of NF-kB dependent iNOS genes[8]. NO production is enhanced in respond to LPS stimuli and mediates the neuroinflammation. Because of the importance of NO derived from IKK activation in inflammatory response, there were several research efforts to find a selective IKK-beta blocker[9,10,11]. Thus, the compounds down-regulating the activity of IKK-beta are suggested to be potential as anti-neuroinflammatory agents.

Using computer aided in silico molecular docking technique and BV2 microglia cell based assay system, we investigated the potential effect of coumestrol as a novel regulator for the IKK-beta-mediated nitric oxide production in microglia. In this study, the structural 3D model of the coumestrol on the IKK-beta protein active site has been performed, which may expedite further development of more natural IKK-beta-protectors to control activated microglia-induced neuronal diseases.

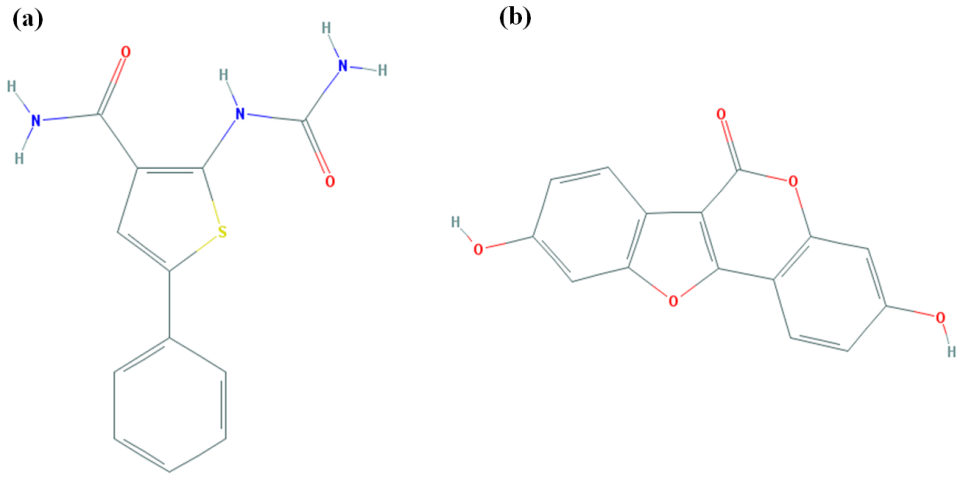

Figure 1. Chemical structure of the (a) IKK-2 inhibitor VI and (b) coumestrol.

MATERIALS AND METHODS

Cell culture

The immortalized murine BV-2 cell line of reactive microglia cells[12] 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[13]. Under a humidified 5% CO_2/95% 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).

IKK beta-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. Coumestrol (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 coumestrol 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 IKK beta protein (PDB ID: 4KIK)(Fig. 1c) was downloaded from the RCSB protein Data Bank. The chemical structure of IKK-2

inhibitor VI (Pubchem CID= 6419765)(Fig. 1a) and coumestrol (Pubchem CID= 5281707)(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[14] to generate atomic coordinates. For docking analysis, PDB coordinates of the target protein, IKK-2 inhibitor VI, and coumestrol molecule were optimized by Discovery Studio version 4.5 software[15]. 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)[16]. A computational ligand-target docking approach was used to analyze structural complexes of the IKK-beta(target) with IKK-2 inhibitor VI and coumestrol (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 IKK-2 inhibitor VI and coumestrol with the IKK-beta 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 coumestrol binding affinity on the IKK-beta was set at *, $p \leq 0.05$ versus IKK-2 inhibitor VI. All data were represented as the means ± SEM (Standard Error of the Means).

RESULTS

Coumestrol binds in high affinity to the IKK-beta protein active site

PyRx Autodock 4 docking analysis was applied to investigate the molecular binding interactions of coumestrol and IKK-2 inhibitor VI molecules, respectively with IKK-beta protein (Fig. 2a) and to elucidate the possible molecular mechanism. As shown in Fig. 2c and Fig. 3b, coumestrol interacted with 9 amino acid residues (LEU21, VAL29, ALA42, LYS44, GLU61, MET96, CYS99, VAL152, ILE165) and IKK-2 inhibitor VI interacted with 7 amino acid residues (TYR107, ASN113, LEU117, ARG118, ALA121, LEU153, GLU378)(Fig. 2b and Fig. 3a). The average molecular binding affinity (docking energy) scores of the IKK-2 inhibitor VI and coumestrol on the IKK-beta target were -6.73 kcal/mol (IKK-2 inhibitor VI, SEM ±0.71) and -9.18 kcal/mol (coumestrol, SEM ±0.86) (Table 1). In addition, the maximum binding affinity of coumestrol (-10.4 kcal/mol) was higher than IKK-2 inhibitor VI (-8.0 kcal/mol) on the IKK beta target protein (Table 1). The numbers of the binding modes of both ligands to the IKK-beta active site were nine (n=9), respectively. Based on the present molecular docking data, coumestrol appeared as a strong binding affinity to the IKK-beta target protein than the IKK-2 inhibitor VI.

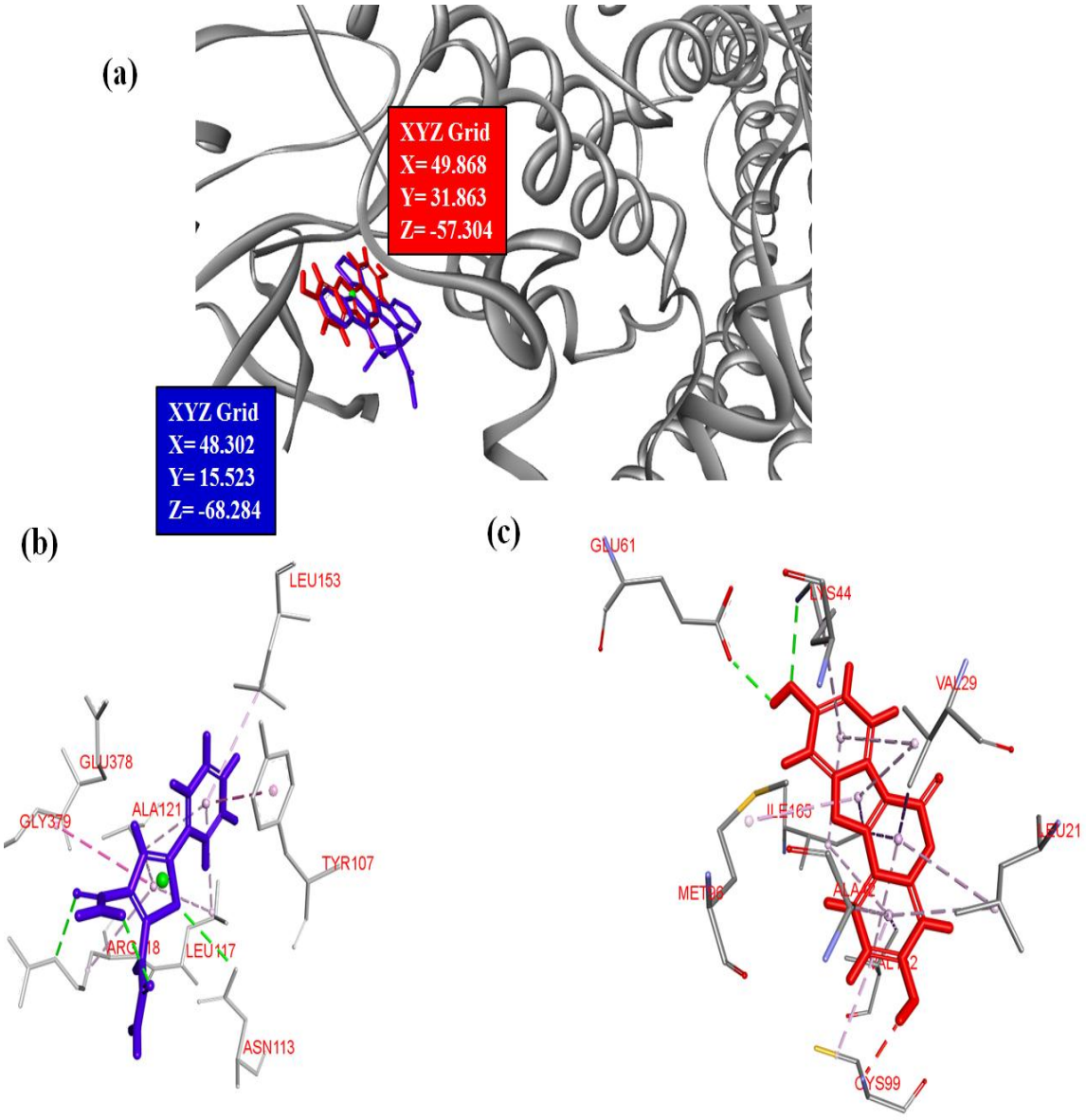

Figure 2. (a) 3D molecular docking pattern and binding position of the IKK-2 inhibitor VI (blue color stick) and the coumestrol (red color stick) on the IKK beta protein A domain (grey color ribbon) active site. (b) 3D pattern of the interacting amino acids with IKK-2 inhibitor VI in the IKK beta protein A domain active site. (c) 3D pattern of the interacting amino acids with coumestrol in the IKK beta protein A domain active site.

Table 1. Binding affinity of the IKK-2 inhibitor VI and coumestrol on the target IKK beta protein active site.

	Max Binding Affinity (kcal/mol)	Average Binding Affinity (kcal/mol)	SEM (n = # of binding mode)
IKK-2 inhibitor VI on the IKK beta protein	-8.00	-6.73	±0.71 (n=9)
Coumestrol on the IKK beta protein	-10.40	-9.18*	±0.86 (n=9)

* $p < 0.05$

Effect of coumestrol on the IKK-beta-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 via IKK-beta – mediation signaling pathway. To elucidate the effect of coumestrol on the IKK-beta-mediated microglial activation, BV-2 cells were incubated with LPS in the absence or presence of coumestrol. Coumestrol significantly reduced LPS-induced NO production in a concentration-dependent manner (Fig. 4), indicating that coumestrol effectively affects TLR-4-IKK iNOS cell signaling.

DISCUSSION

Coumestrol is well known phytoestrogen and has a planner(polyphenol) structure(fig. 1b) favorable to enter into active site of target protein effectively and has shown strong affinity with IKK-beta protein binding pocket(Fig. 2a, Fig. 2c, Fig 3b and Table 1). The computational docking of IKK-beta target with coumestrol using auto docking procedure revealed that all the lowest energy complexes of IKK-beta are stabilized by intermolecular aromatic ring-mediated many pi-alkyl interactions (12 interactions) and coumestrol additionally makes unfavorable donor-donor, pi-sigma interactions (Fig. 3b) than IKK-2 inhibitor VI does (5 pi-alkyl interactions)(Fig. 3a). The calculated final docked maximum binding affinity of coumestrol to IKK-beta protein is −10.40 kcal/mol and IKK-2 inhibitor VI (standard) is −8.00 kcal/mol (Table 1). Therefore, docking results revealed that this coumestrol compound can enter the key active site of the IKK-beta protein and interacts effectively than IKK-2 inhibitor VI. The interacting numbers of the chemical bonds and binding patterns of the ligand among the IKK-beta amino acids might be critical factors for coumestrol to regulate target IKK-beta protein activity. These data strongly 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 IKK-beta and its interacting ligands, coumestrol.

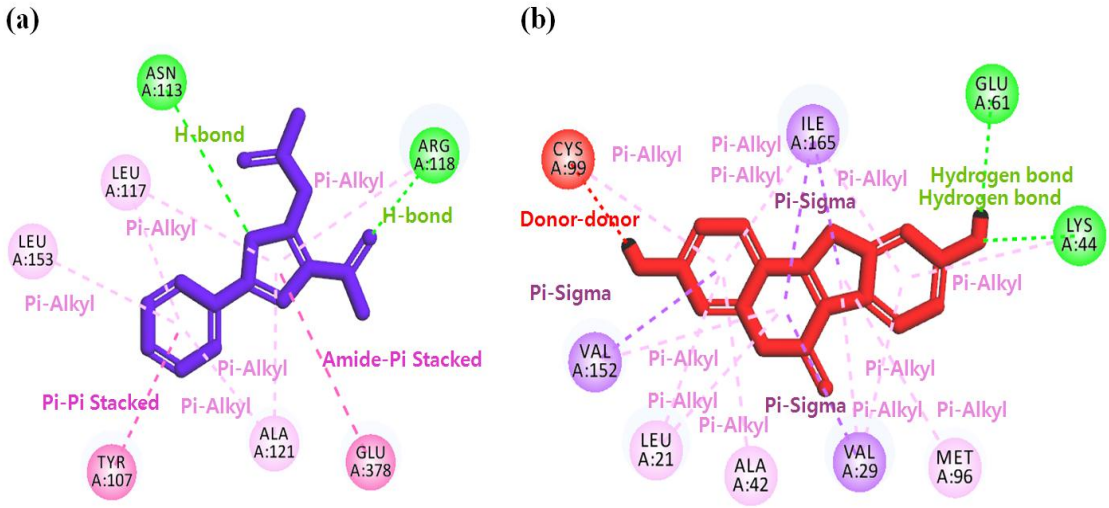

Figure 3. (a) The name of the chemical bonds (or interactions) and 2D pattern of the interacting amino acids with IKK-2 inhibitor VI in the IKK-beta active site. (c) The name of the chemical bonds (or interactions) and 2D pattern of the interacting amino acids with coumestrol in the IKK-beta active site.

Neuroinflammation is one of the main brain reactive mechanisms involved in the progression of dangerous neurodegenerative diseases, such as Alzheimer, Parkinson, multiple sclerosis, amyotrophic lateral sclerosis[17]. Among these, microglia act as resident brain macrophages that become inflammatory activated in most brain pathologies[18]. 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[18].

Natural herb derived flavonoids, possess neuroprotective potential probably related to their ability to regulate the neuroinflammatory responses involved in neurodegenerative diseases[19,20]. Coumestrol (or coumestrol enriched soybean 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[17,21]. The aim of the present study was to evaluate the effect of coumestrol, one of the most abundant flavonoids in soybean, on IKK-beta-mediated NO production in BV2 microglia. Several previous studies have revealed that anti-neuroinflammatory effects of natural flavonoids in neurodegenerative disorders[17,22,23,24]. It was noteworthy that coumestrol pretreatment significantly protected LPS-TLR4-IKK-beta-signal pathway mediated neuroinflammation in microglia cell system(Fig. 4). In accordance with the previous reports[25,26], our data also demonstrated that coumestrol effectively protected IKK-beta-mediated nitric oxide production and neuroinflammation (Fig. 4).

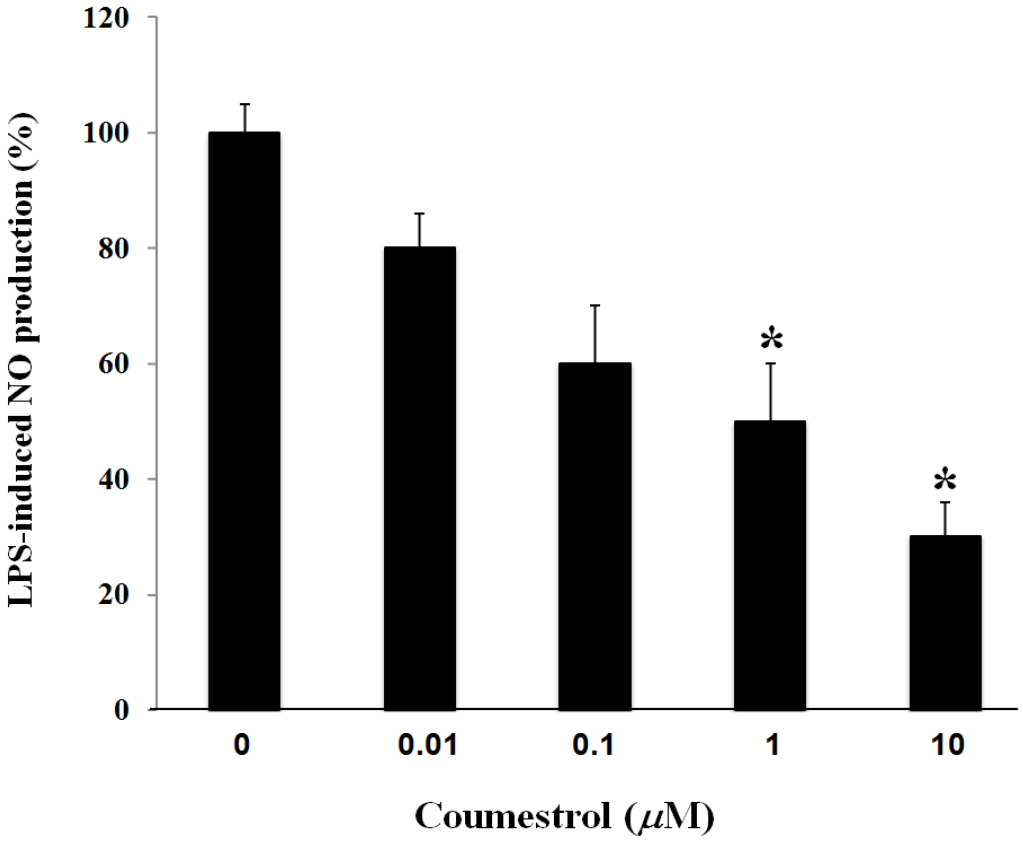

Figure 4. Effects of coumestrol on the NO production induced by LPS in BV-2 microglial cells. Data represent means ± SEM of three independent experiments. Significant differences between LPS and quercetin + LPS are presented. *$p < 0.05$.

CONCLUSION

These experimental results presented here highlight the potential therapeutic use of coumestrol as neuroprotective agents and demonstrated clearly that coumestrol accurately and effectively interact with IKK-beta protein target. Therefore, coumestrol might play an important role in inhibiting IKK-beta protein mediated nitric oxide production and neuroinflammation. In conclusion, coumestrol can play an important role in altering the progression of neuroinflammatory diseases by its protective effect against IKK-beta protein mediated oxidative brain stress.

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