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Comparative molecular docking studies of lupeol and lupenone isolated from *Pueraria lobata* that inhibits BACE1: Probable remedies for Alzheimer's disease

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ABSTRACT

Objective: To discover lead lupane triterpenoid's potential isolated from *Pueraria lobata* roots against β-site amyloid precursor protein cleaving enzyme 1 (BACE1), which serve as a rate limiting step in amyloid beta (Aβ) production altering the course of Alzheimer's disease. In addition, enzyme kinetics study and molecular docking were conducted to establish the inhibition type and structure activity relationship.

Methods: A systematic study of 70% ethanolic *P. lobata* root extract was employed to identify its BACE1 inhibitory potential. Further, BACE1 inhibitory potential of two lupane terpenoids, yielded from ethanolic extract, was assessed. In order to determine their inhibition mode, Lineweaver–Burk plots and Michaelis–Menten model for BACE1 was performed. AutoDock 4.2 program in addition determined the molecular interaction of BACE1 with isolated terpenoids.

Results: Considering the inhibitory potential of 70% ethanolic extract *of P. lobata* against BACE1 (IC_{50} = 80.35 μg/mL), lupeol and lupenone were subsequently isolated and exhibited notable or moderate BACE1 inhibitory activity with IC_{50} values of 5.12 and 62.98 μmol/L, respectively, as compared to the positive control quercetin (IC_{50} = 21.28 μmol/L). The enzyme kinetics study enabled us to identify both compounds as competitive inhibitors, where lupeol displayed a very potent inhibition against BACE1 with low inhibition constant (K_i) value of 1.43 μmol/L, signifying greater binding affinity. In order to understand the binding mechanism and structure–activity relationship of two triterpene-based BACE1 inhibitors, we employed computer aided docking studies which evidently revealed that hydroxyl group of lupeol formed two hydrogen bonds with the ASP32 (catalytic aspartic residue) and SER35 residues of BACE1 with the binding energy of (−8.2 kcal/mol), while the ketone group of lupenone did not form any hydrogen bonds with BACE1 giving evidence for less binding affinity. These results in turn have predicted the dependence of the inhibitory activity in the presence of hydroxyl group which has provided a new basis for BACE1 blockade.

Conclusions: Our results have successfully explored the molecular mechanism of lupane triterpenoids via BACE1 inhibition, suggesting that lupeol in particular could be utilized as a useful therapeutic and preventive agent to mitigate Alzheimer's disease.

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1. Introduction

Generally, the accumulation of amyloid beta (Aβ) in fibrillar plagues and soluble oligomers in the higher regions of the brain defines Alzheimer's disease (AD). AD is postulated to be characterized by intracellular neurofibrillary tangles, neuroinflammation, and neuronal dysfunction leading to death. Cumulatively, Aβ is considered the hallmark of AD responsible for triggering a complex pathological cascade leading to neurodegeneration [1]. Further, β-site amyloid precursor protein cleaving enzyme 1 (BACE1) controls the rate limiting step in the production of Aβ responsible for the pathogenesis of AD, which has sought the researchers to target BACE1 for the mitigation of AD [2]. Protein levels of BACE1 are significantly higher in patients having AD, which explains the high importance being given to BACE1 inhibition [3].

Recently, natural products-derived lead compounds for the treatment of AD have increased, as they are free of any potential life threatening side effects. Despite the availability of U.S. Food and Drug Administration approved drugs, like donepezil, tacrine, rivastigmine, and galantamine, for the symptomatic treatment of AD; observable toxic issues such as hepatotoxicity, vomiting, diarrhea, and nausea have forced these drugs out of the pharmaceutical market [4]. *Pueraria lobata* (Wild.) Ohwi (*P. lobata*) also known as kudzu, is a perennial vine native to Asia, primarily subtropical and temperate regions of China, Japan, and Korea, whose roots can account for up to 40% of the total plant biomass [5,6]. The starch extracted from its roots is used as herbal medicines, and foods, including naengmyeon. In China, the root is used in herbal remedies and mostly in teas. Recent research has focused on a kudzu root-derived medicinal product for alcohol-related problems [7]. *P. lobata* roots are known to exhibit antioxidant [8], anti-inflammatory [9], hepatoprotective [10], anti-diabetic [11], anti-dipsotropic [12], anti-atherogenic activities [13], hypolipidemic [14], and anti-obesity [15]. Kudzu also has compounds that display estrogenic activity [16] and is used for cardiovascular treatment [17]. A group of terpenes with particular importance are triterpenes that have been identified and classified according to their structures and chemical properties, and can be found in the form of aglycones or as free acids. Emphasizing from a biological perspective, the most important triterpenes are the pentacyclic oleanane, ursane, and lupane that are abundant in higher plants [18]. Lupeol and lupenone from *P. lobata* are pentacyclic triterpenes of 30-carbon skeleton, comprising 4 six membered rings and 1 five membered ring [19,11]. Substantial research over the last three decades has uncovered several important pharmacological activities of lupeol, establishing it as a magical drug. Lupenone and lupeol have been accounted with diverse bioactivities, including anti-inflammatory [20], antioxidant [21], antitumor [22], immunomodulatory [23], antileishmanial [24], and antibacterial [25]. Despite several efforts have been attempted to clarify the pharmacological activities of lupeol and lupenone, the stereochemistry on BACE1 have not been characterized yet. Thus, the knowledge on structure activity relationship (SAR) of terpenoids interacting with BACE1 is vital to understand the enzyme target. Our study aimed to provide the comparative inhibition effect of lupeol and lupenone against BACE1 highlighting its importance. Only limited researches regarding the stereochemistry of lupeol have been attempted. Binding mode on protein tyrosine phosphatase 1B [26], urinary tract infection [27], and cancer [28] have been demonstrated by various literature. Therefore, the molecular basis for binding to the active site of BACE1 is elucidated via computer aided molecular binding analysis, which is believed to be the first of its kind regarding BACE1.

2. Materials and methods

2.1. Chemicals and reagents

A BACE1 FRET assay kit was purchased from PanVera Co. (Madison, WI, USA). All the required reagent-graded chemicals used in this study were bought from commercial sources.

2.2. Plant materials

P. lobata were gathered from Gangwon-do Province, Korea, in March 2015, and validated by Prof. Jae Sue Choi. A voucher specimen (20150320) was deposited in the Prof. Choi's lab.

2.3. Extraction and fractionation

The dried root of *P. lobata* (1 kg) after extraction with distilled water gave 175 g of extract. The dried root of *P. lobata* (3 kg) was again separately extracted with 70% ethanol (EtOH) under reflux, yielding 0.9 kg of extract. The 70% EtOH extract was then fractionated with different soluble solvents to yield *n*-hexane (27.5 g), dichloromethane (4.3 g), ethyl acetate (22.2 g), and *n*-butanol (391 g) fractions, as well as water residue (455 g).

2.4. Isolation of compounds

Lupenone (234 mg) and lupeol (550 mg) were isolated from the *n*-hexane fraction (27.5 g), that were elucidated via some spectroscopic methods, including proton and carbon-NMR, as well as through the published spectral data [11,29].

2.5. In vitro BACE1 enzyme assay

Assays were performed using the commercial protocol, BACE1 FRET assay kit (PanVera Co.) method with slight modification. Quercetin was used as a standard.

2.6. Type of inhibition of lupeol and lupenone towards BACE1 using enzyme kinetics

To determine the kinetic mechanisms of lupeol and lupenone towards BACE1, we produced Lineweaver–Burk plot and Michaelis–Menten model by varying concentrations of substrate (0–750 nmol/L) and inhibitors (0–14 μmol/L for lupeol and 0–120 μmol/L for lupenone) [30]. Kinetic parameters, including inhibition constants (K_i), maximum reaction velocity (V_{max}), and Michaelis Menten constant (K_m) values were calculated via Sigmaplot 12.0 (Systat Software Inc., San Jose, CA) [31,32].

2.7. Molecular docking simulations

AutoDock 4.2 software was employed to assess the structure of the enzyme-inhibitor complex. In our study, lupeol and lupenone were tested for BACE1 inhibition. AutoDock 4.2 predicts binding free energies of enzyme-inhibitor complexes and the

binding energies of both the bound and unbound states using semi empirical free energy force field. The 3D structures of the BACE1 (2WJO) were acquired from RCSB Protein Data Bank. The 3D structures of lupeol, lupenone were obtained from Pubchem Compound (NCBI) (259846 and 92158, respectively). The automated docking model was generated using AutoDock Tool [33,34]. The co-crystallized ligand, 2-amino-3-{(1*R*)-1-cyclohexyl-2-[(cyclohexylcarbonyl)amino]ethyl}-6-phenoxyquinazolin-3-ium (QUD), was used to generate the grid box for catalytic inhibition mode. The grid box size was 60 × 60 × 60 and the x, y, z, center was 26.593, 41.585, 41.026. PyMOL 1.7.4 and LigPlot^{+} were employed to obtain the number of hydrogen bonds and van der Waals interacting residues.

2.8. Statistical analysis

ANOVA and Student's *t*-test were used to analyze statistics. Data are presented as the mean ± SEM of at least four independent experiments.

3. Results

3.1. Anti-AD potential of P. lobata roots extracts

The anti-AD potentials of the 70% EtOH and H_2O extracts were evaluated against BACE1 inhibition. The results obtained are shown in Figure 1. Additionally, the 70% EtOH extract demonstrated modest BACE1 inhibitory potential (IC_{50} = 80.35 ± 3.13 μg/mL), compared with quercetin (IC_{50} = 5.58 ± 0.10 μg/mL). This showed the BACE1 inhibitory potential of 70% EtOH fraction.

3.2. Anti-AD potential of lupeol and lupenone

Bioassay-guided isolation yielded two compounds from the 70% EtOH extract. We then investigated their BACE1 activities. Lupeol showed potent BACE1 activity with an IC_{50} value of (5.12 ± 0.30) μmol/L, whereas, lupenone with an IC_{50} value of (62.98 ± 2.22) μmol/L showed moderate inhibition compared to the positive control, quercetin [(21.28 ± 1.42) μmol/L]. Consequently, we further focused on their enzyme kinetic analysis and molecular docking study.

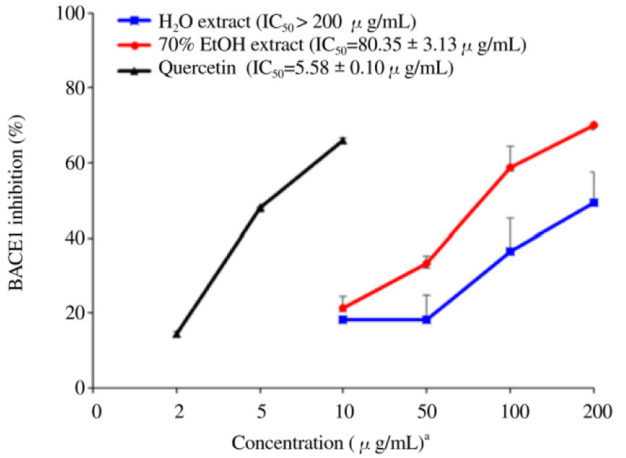

Figure 1. Concentration-dependent BACE1 inhibitory activities of H_2O and 70% EtOH extracts of *Pueraria lobata* roots along with the standard, quercetin.
Error bars indicate standard error of the mean (SEM). [a]The 50% inhibitory concentration (IC_{50}) values (μg/mL) were calculated from a log dose inhibition curve and expressed as mean ± SEM of triplicate experiments.

3.3. Enzyme kinetic analysis of lupeol and lupenone for BACE1

Lineweaver–Burk plot and Michaelis–Menten model were used to determine the type of enzyme kinetics. The slopes or the intercepts in the Lineweaver–Burk plots were drawn in SigmaPlot 12. For BACE1, lupeol and lupenone showed competitive modes of inhibition as shown in Table 1. Lupeol showed the utmost potent binding affinity with a K_i value of 1.43 μmol/L. Likewise, the K_i value for lupenone was higher, showing relatively weaker binding affinity, leading to the conclusion that a higher concentration is required to inhibit enzyme activity. All the results associated to K_m, V_{max}, and K_i are included in Table 1.

3.4. Molecular docking simulation studies of lupeol and lupenone on BACE1 enzyme

Molecular docking studies were used to estimate the enzyme-inhibitor interaction geometrics for the selected compounds. Table 2 demonstrates the docking scores for lupeol and lupenone with interacting BACE1 residues including hydrogen bond and van der Waals interacting residues. It was discovered that the potential of lupeol and lupenone against BACE1 was linked with the binding energy and the number of hydrogen bonds formed in the catalytic site.

The hydroxyl group of lupeol formed two hydrogen bonds with the BACE1 catalytic residues, ASP32 (catalytic aspartic residue) and SER35, and had a binding energy of −8.63 kcal/mol for BACE1 (PDB ID: 2WJO) (Figure 2A and C and Table 2). Further, we noted that the van der Waals interaction of lupeol with TYR71, GLN73, TRP76, LYS107, PHE108, and ILE118 further stabilized the enzyme-inhibitor interaction. In contrast, the ketone group of lupenone did not form any hydrogen bonds with BACE1. However, the molecular docking study revealed that the ASP32, SER35, VAL69, TYR71, LYS107, PHE108, ILE110, and GLY230 residues were involved in van der Waals interactions with lupenone as shown in Figure 2(B and D) and Table 2.

Table 1
Kinetic parameters of BACE1 inhibition by different concentrations of lupeol and lupenone.

Inhibitor	Concentration (μmol/L)	Substrate (Rh-EVNLDAEFK-Quencher)			
		K_m [a]	V_{max} [b]	K_i [c]	Inhibition mode[d]
Lupeol	2	1997	2.07	1.43	Competitive
	7	4062	2.00		
	14	11890	2.95		
Lupenone	12	1624	1.03	40.41	Competitive
	60	2919	1.07		
	120	4887	1.05		

[a] Michaelis Menten constant (K_m) with free enzyme. [b] Maximum reaction velocity (V_{max}). [c] Inhibition constant (K_i). [d] Determined using Lineweaver–Burk plot and kinetic parameters.

Table 2
3D docking interaction of BACE1 (2WJO) with lupeol and lupenone from *P. lobata* as well as the reported catalytic ligand, QUD.

Compounds	Binding energy (kcal/mol)	Interaction residues (amino acids)
Lupeol	−8.2	H-bond (2): Asp32, Ser35 van der Waals: Tyr71, Gln73, Trp76, Lys107, Phe108, Ile118
Lupenone	−7.8	H-bond (0): - van der Waals: Asp32, Ser35, Val69, Tyr71, Lys107, Phe108, Ile110, Gly230
QUD	−9.3	H-bond (4): Asp228, Asp32, Gly230 van der Waals: Ile118, Tyr71, Gly74, Lys75, Lys107, Val69, Ile226, Thr231, Thr329, Gly34, Tyr198, Arg235, Leu30, Ser35

QUD: 2-amino-3-{(1*R*)-1-cyclohexyl-2-[(cyclohexylcarbonyl)amino]ethyl}-6-phenoxyquinazolin-3-ium.

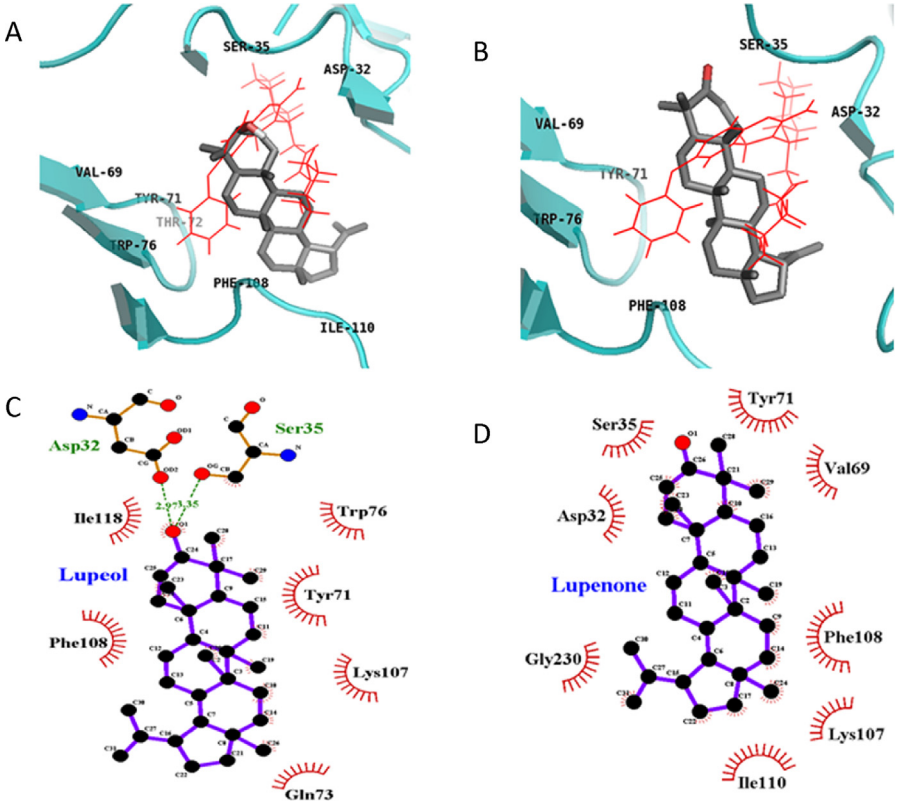

Figure 2. Inhibition mode of lupeol (A), lupenone (B) for the BACE1 catalytic site with QUD (red line) and 2D ligand interaction diagram of BACE1 inhibition by lupeol (C) and lupenone (D).
Dashed lines indicate H-bonds. Carbons are in black, nitrogens in blue, and oxygens in red. The figure was generated using PyMOL and LigPlot^{+}. QUD: 2-amino-3-{(1*R*)-1-cyclohexyl-2-[(cyclohexylcarbonyl)amino]ethyl}-6-phenoxyquinazolin-3-ium.

4. Discussion

BACE1 being the rate-limiting step of Aβ formation from its β-site amyloid precursor protein (β-APP) are being considered and investigated in various clinical trials for their ability to lessen cerebral Aβ concentrations to prevent AD [34]. A 70% EtOH extract of *P. lobata* roots exhibited potent anti-diabetic effects in our previous work [11]. In an attempt to extend our research to understand its anti-AD activity, we subsequently revealed its modest likelihood to inhibit BACE1. Given this potential, further fractionation and isolation yielded lupane triterpenoids, lupeol, and lupenone. Lupeol is an important structural component of plant membranes [35] that has been reported to attenuate lipopolysaccharide-induced neuroinflammation in mouse brains [36], and also has pharmacologically prevented cell death caused by glutamate or $A\beta_{(25–35)}$ protein, even at 10 μmol/L [37]. Based on the literature survey, lupeol was not found to be targeted against BACE1, rather, was found to have a potential to inhibit acetylcholinesterase/butyrylcholinesterase [38]. Another report has mentioned lupeol's ability to minimize $A\beta_{(1–42)}$ induced dementia, proposing its potential to therapeutically treat AD like symptoms [39]. Thus, to elucidate the activity against major target BACE1, we thrived our studies in which lupeol showed potent BACE1 activity with an IC_{50} value of 5.12 μmol/L and was consequently even more potent than the positive control, quercetin (21.28 μmol/L); being the first to report on BACE1 inhibition. Amyloid cascade and cholinergic hypotheses are two major hypotheses regarding AD. Among them, researchers have visualized BACE1 as an effective mode to control AD, which is responsible for the formation of Aβ. On the contrary, acetylcholinesterase and butyrylcholinesterase inhibitors act by recovering the cholinergic transmitter level acting as a marker to predict the development of AD. Besides, various studies have focused BACE1 as an excellent target against AD supporting amyloid cascade hypothesis [40–42]. Thus, the exploration of BACE1 inhibitors seems vital for AD treatment. Another terpenoid, lupenone, showed relatively weaker activity

(62.98 μmol/L) as compared to lupeol which might be attributed to their chemical structures. Hydroxyl (OH) group at C-3 might play a pivotal part in the activity as suggested by the potent cytotoxic activity [43], which also explains the importance of OH moiety for the activity. Our study also demonstrated the potential inhibitory property of lupeol which might be directed to its configuration. Thus, due to lupeol's active neuroprotective ability, our study focused on its kinetic and molecular study to pave the potential reaction mechanism.

The Lineweaver–Burk plot is widely used in enzyme kinetic studies to examine whether the compounds inhibited BACE1 activities by interacting with the enzyme's active site or not. In our present study, lupeol displayed a competitive type of inhibition, with very potent inhibition against BACE1, correlated to its low K_i value (1.43 μmol/L), signifying greater binding affinity. Lupeol has been demonstrated as a competitive ligand-binding inhibitor proving as a potent androgen receptor inhibitor preventing hormone refractory diseases [44]. On the other hand, lupenone displayed competitive inhibition with relatively high K_i value. Previous reports have demonstrated lupane terpenoids as a non-competitive protein tyrosine phosphatase 1B inhibitors for drug design [11,45]. Our study has determined that both lupane triterpenes inhibited in a competitive manner.

Current study employed molecular docking simulation study visualizing the reaction between a protein and a small molecule at the molecular level. By this, we can predict their behavior in the binding site of target proteins interpreting the ultimate biochemical processes [46,47]. Given the goal of molecular docking to be able to predict binding affinities, it also allowed us to confirm and compare the BACE1 inhibitory activities of the isolates and the inhibition mode was acquired from chemical kinetics. Based on the docking energy and a good interaction with active site residues, the docked ligand molecules were selected. The 3D docking of lupeol and lupenone exhibited a minimum docking score for BACE1. The lower the docking score, the greater is the binding capacity of the ligand. Hence, the docking scores and binding interactions of compounds from *P. lobata* were expressively associated with their capability to inhibit the activity of BACE1. The OH group of lupeol formed two hydrogen bonds with the ASP32 and SER35 residues of BACE1, while the ketone group of lupenone did not form any hydrogen bonds with BACE1. In addition, the binding energy of lupeol (−8.2 kcal/mol) was lower with a higher binding affinity than that of lupenone (−7.8 kcal/mol). These *in silico* docking results were in good agreement with the *in vitro* experimental data. In particular, this study is the first report of the BACE1 inhibitory activity of lupeol and lupenone derived via enzyme kinetic analysis and molecular docking simulation.

In addition, to define the inhibitory effect of the lupane type terpenoids against BACE1, we sought to establish a SAR between the compounds and target enzyme inhibition. Among the lupane triterpenoids investigated, lupeol had the highest inhibitory potential against BACE1 which can be attributed to its OH group. The absence of an OH group diminished the inhibitory potential as evidenced in lupenone. Based on the above SAR, the inhibitory activity appears to be largely dependent on the presence of OH group. The polar functional group in lupenone was a carbonyl group which might reduce the interaction. The activity of the triterpenoids thus seem to be depending on the substituent at C-3; in the order of, OH > C═O. The vitality of the presence of OH group has been evidenced in our previous study [48]. A recent study on one of the lupane triterpenoid, betulinic acid exhibited moderate BACE1 interference activity dependant upon the conformation of the C-3 OH group. Upon inverting C-3 OH group yielded more potent congeners. So, confirmation of the C-3 OH group was vital along with the free OH group [49]. Our study also showed the importance of C-3 hydroxy group in the BACE1 inhibitory activity. With respect to the ability to inhibit BACE1 activity, lupeol and lupenone had comparably similar potent inhibitory potential, which further provides potential strategies for the design of BACE1 inhibitors. Thus, the SAR of lupeol–lupenone combined with the molecular docking studies have enabled us to know the important factors that play role in the binding of lupeol with the active site of BACE1. These results provide the basis for lupane type triterpenoid's interaction with BACE1 that may develop a new potential lead for BACE1 blockade. Moreover, it also accounts for the molecular basis required for various natural products containing lupeol type terpenoids for the prevention of AD.

P. lobata showed significant inhibitory activity against BACE1, a key enzyme responsible for AD; attributed to the isolated lupeol triterpenoid. Our study identified lupeol as a potent BACE1 inhibitor confirmed via enzymatic experiments and the docking simulation. Therefore, a new potent BACE1 inhibitor, lupeol, has been discovered that holds promise as a therapeutic component for treating AD.

Conflict of interest statement

The authors declare they have no conflict of interest.

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