Identification of Ent-Kaurane Diterpenoid Compounds as Potential Inhibitors of the PI3K Pathway in Nonsmall Cell Lung Cancer Through Molecular Docking Simulations

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Abstract
Annual mortality of 8.2 million could be attributable to cancer globally, posing a serious health issue; particularly, the high number of nonsmall cell lung cancer (NSCLC) diagnosed cases in recent years highlight the need for development in anticancer agents. In NSCLC, a number of specific inhibitors of phosphatidylinositol-3-kinase (PI3K), Protein kinase B (AKT), and mammalian target of rapamycin are currently under development; however, the early evidence has yielded disappointing results. Ent-kaurane diterpenoid compounds from Cronton tonkinensis have been investigated for several bioactivities such as antibacterial, cytotoxic activity, and so on; however, lung cancer is not yet studied. In this study, we conducted a molecular docking study of 7 ent-kaurane diterpenoids from C. tonkinensis against PI3K targeted anticancer therapies; furthermore, their cytotoxicity effects against A549 lung cancer cells were also evaluated. Obtained results indicated that compounds 7, 6, 2, and 1 exhibited significant inhibitory results in comparison to the reference drug oxaliplatin which suggests further in vitro assay for drug development.

Keywords
ent-kaurane diterpenoids, molecular docking, lung cancer, phosphatidylinositol-3-kinase pathway

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Introduction
Approximately 1.79 million deaths could be attributed to lung cancer annually, making it one of the most prevalent cancers worldwide, particularly in Eastern Asia.¹,² Lung cancer often manifests as heterogeneous tumors, in which nonsmall cell lung cancer (NSCLC) occurs in ~70% to 85% of patient cases.³ Despite many efforts that have been made to improve the systematic therapies of NSCLC patients, the rate of survival for the patients is only around 15%.³,⁴ Currently, combinational treatment of NSCLC involving surgery, chemotherapy, and radiotherapy is suggested to be inadequate to improve the prognosis for the patient.³ Alternatively, patients seek possible treatment in alternative medicines, most notably traditional medicine. In Vietnam, traditional medicine involving the use of herbs and therapies has been widely adopted since a thousand years; it is well established and grounded with various scientific evidences regarding the treatment of various diseases including cancer.⁵,⁶,⁷ Croton tonkinensis Gagnep. (Euphorbiaceae), or commonly referred to as “Kho sam cho la” in Vietnamese, is a native plant in the north of Vietnam.⁸ It is reported that ent-kaurane diterpenoids from C. tonkinensis possess a myriad of biological effects including inhibition of silent information regulator 2 ortholog 1, stimulation of osteoblast differentiation, and anti-inflammatory, antibacterial, or antitumor activity.⁹-¹² Of the various ent-kaurane diterpenoids from C. tonkinensis described

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so far in the literature, only a few compounds have been studied against the NSCLC model; therefore, the mechanism by which they affect this cell line is still scarcely explored.10,13-15

In NSCLC, the phosphatidylinositol-3-kinase (PI3K) pathway has been heavily implicated in both tumorigenesis and the progression of disease.16 There are various genomic episodes that could distort the normal regulation of the pathway such as mutation, amplification, and displacement. In addition, the pathway could be affected by growth factors and regulators to give enhanced metastatic suitability and improved resistance to therapies.17 The PI3K target proteins are divided into classes and subclasses based on their functions and structures in which class 1A is highly accountable for human cancer development. It has been reported that the activation of this class causes a signaling protein phosphoinositide-dependent kinase-1 (PDK1) to promoted the protein Protein Kinase B (AKT);18-20 thus, mouse double minute 2 homolog (MDM2) movement to the nucleus could also be facilitated ultimately restricting MDM2 to the cytoplasm by undermining p52 functioning (Supplemental Figure S1).21-23 This suggests that the lack of feedback inhibition causes activation of upstream proteins, including AKT, solely through inhibition of the mammalian target of rapamycin (mTOR). These notions raise the need for multiple site targeting when it comes to intervening in the inflammatory pathway, which involves arachidonic acid and its metabolism, operating through transformations from cyclooxygenases (COXs) to prostaglandins.29 This process improves AKT and the aforementioned downstream signals; in addition, previous studies have shown that the tumor multiplicity is inversely correlated with inhibition of 5-lipoxygenase (5-LOX) and that greater effectiveness could be achieved with simultaneous inhibition of both 5-LOX and COX-2.30,31 Currently, there have been continuous efforts conducted by scientists in developing novel agents that specifically inhibit PI3K pathway targets such as gefitinib, perifosine, sirolimus, and so on. Several of them have processed to the clinical trials stage; however, the early evidences showed disappointing results.32,33

In this study, 7 ent-kaurane diterpenoid compounds originated from C. tonkinensis were selected for molecular docking simulation to investigate potential inhibition activities with different targets of the PI3K pathway; in addition, their in vitro cytotoxicity effects against human lung cancer cell line A549 were then assessed. The simulated and experimental results are expected to contribute to enhance cancer therapy and guide further in vitro and in vivo studies.

Results and Discussion

Molecular Docking Study

Molegro Virtual Docker (MVD)34 and Ligplot + v2.135 were utilized to give insight into the orientation of the molecule at the active site of a targeted protein and identify residues participating with a ligand. In the docking results, detailed analysis including binding affinity calculation and hydrogen bond interaction were made for the top-ranked dock pose of each compound.

Docking score analysis revealed that among the studied molecules, compound 6 was recorded as the topmost when binding to an mToR active site at −124.5 kcal/mol, followed by compound 1 for COX-2 enzyme at −110.2 kcal/mol. Regarding the hydrogen bonding patterns, the former exhibited no hydrogen bonding, suggesting that the ligand–protein interaction is unstable, while the latter showed H-bond interactions with Phe210, Gln289, and Tyr385 mediated by its O17 and H1 atoms. Similarly, binding affinity calculations showed that compounds 7 and 1 have the highest affinity towards mToR active sites with the corresponding values of −16.1 and −15.8 kJ/mol, respectively. In general, regarding binding affinity against a target, compound 7 was found to exhibit the best affinity to 4 out of 5 targets (AKT, mToR, MDM2, and PDK1), while COX-2 showed the best binding affinity to compound 6. The details of the interaction are displayed in Table 1, Figure 1, and Supplemental Figures S2 to S5.

Comparing results with the reference compound, we found that for the AKT model, 4 compounds recorded 0 have higher docking scores than oxaliplatin including compounds 1, 7, 6, and 2 with corresponding values of −85.9, −84.0, −81.4, and −80.3 kcal/mol, respectively. Although oxaliplatin did not form hydrogen bonds to AKT protein, it is observed that the 4 mentioned compounds created the same interaction with Glu 114, and compound 7 was proved to have the most stable interaction as evidenced by a total of 7 formed hydrogen bonds. Regarding the second target, docked results with the mToR model showed that compounds 6, 2, and 1 exhibit better binding free energy than oxaliplatin even though residues targeted by the compounds were not quite on a par with the drug. In addition, compound 7 is highlighted due to its highest binding affinity (−16.1 kJ/mol) to mToR through H-bond interaction with Val2227. Considering interaction with COX-2, it should be noted that all the compounds consistently scored low as compared to the reference with regard to both docking scores and binding affinity. In this case, compound 6 is considered to have potential based on its dock score and the number of hydrogen bonds formed with essential residues in comparison to oxaliplatin, such as Asn382 and His386. For the MDM2 target, compounds 7 and 6 showed better docking scores than the rest (−92.8 and −104.5 kcal/mol, respectively); however, only compound 7 and oxaliplatin shared common hydrogen bond interaction with Leu50, which suggests it might be the molecule with most potential to inhibit this target. Finally, the results with the PDK1 target showed that compounds 6, 1, and 7 showed better docking scores than that of the reference oxaliplatin (−101.0, −91.8, and −87.9 kcal/mol, respectively). In a more detailed analysis, compound 7 formed 8 hydrogen bonds in the binding site with PDK1 and binding affinity reached up to −15.0 kJ/mol; thus, it is assumed as a potential candidate for this target.
Table 1. Docking Scores of *Ent*-Kaurane Diterpenoids and Oxaliplatin Against AKT, mToR, COX-2, MDM2, and PDK1 Protein Targets.

| Protein target | Ligand | Docking score (kcal/mol) | H-bonding residues | Binding affinity (kJ/mol) |
|----------------|--------|--------------------------|-------------------|--------------------------|
| AKT (PDB id: 1UNQ) | Compound 7 | −84.0 | O28-O-SER56, O28-O-LEU78, O18-ALA58, O25-O-LEU110, O25-LEU110, O25-GLU114, O23-OSER56 | −13.2 |
| | Compound 6 | −81.4 | O25-GLU114, O25-LEU110, O29-ALA58, O29-SER56 | −12.5 |
| | Compound 2 | −80.3 | O25-GLU114, O25-LEU110, 029-ALA58, O29-SER56 | −11.9 |
| | Compound 4 | −54.3 | O25-O-ASP32, O23-O-LEU110, O17-O-GLN104, O22-O-CYS60 | −10.0 |
| | Compound 3 | −53.4 | O22-O-ASP32 | −8.9 |
| | Compound 5 | −56.3 | O25-O-GLN59, O25-N-LYS111, O25-O-GLU114 | −10.7 |
| | Compound 1 | −85.9 | O24-O-GLU114, O24-N-GLN59, O24-N-LYS111 | −12.7 |
| Reference Oxaliplatin | | | nil | −18.5 |
| mToR (PDB id: 4JSV) | Compound 7 | −72.3 | O18-N-VAL2227 | −16.1 |
| | Compound 6 | −124.5 | NIL | −14.4 |
| | Compound 2 | −104.9 | O25-N-VAL2227, O18-O-VAL2227, O23-O-GLU2196, O18-O-GLU2196 | −13.2 |
| | Compound 4 | −84.7 | O18-O-GLU2196 | −10.5 |
| | Compound 3 | −84.8 | O8-O-GLU2196 | −11.6 |
| | Compound 5 | −89.8 | O22-O-LEU1936, O22-O-GLN1937, O18-N-GLN2200 | −10.5 |
| | Compound 1 | −108.7 | O24-N-THR2143, O24-N-TYR2144, O18-N-VAL2227, O8-O-GLU2196 | −15.8 |
| Reference Oxaliplatin | | | O13-O-HIS386, O14-O-HIS386, O13-O-ASN382, O13-O-ASN382, N6-O-TYR385 | −21.8 |
| COX-2 (PDB id: 3NTI) | Compound 7 | −108.8 | O13-O-PRO2229, O14-O-PRO2229, H64-ON-HIS388, O25-N-GLN289, O18-O-PHE210, O25-O-PHE210, O18-N-HIS207 | −20.0 |
| | Compound 6 | −109.5 | O17-N-HIS207, O17-O-PHE210, O29-O-THR212, O23-O-THR-212, O29-N-ASN382, O23-N-N-ASN382, O18-O-THR212, O23-O-TYR148 | −14.8 |
| | Compound 2 | −89.7 | O25-O-ASN382, O25-O-HIS386, O18-N-HIS207, O18-N-GLN203 | −11.6 |
| | Compound 4 | −81.7 | O22-N-THR212, O22-O-THR212, O17-N-HIS214, O18-N-GLN289 | −11.5 |
| | Compound 3 | −81.5 | O18-O-PHE210, O18-N-GLN289, O17-N-HIS214, O22-N-THR212, O22-N-THR212 | −10.9 |
| | Compound 5 | −107.3 | O17-O-PHE210, O17-N-GLN289, O25-C-HIS386 | −10.4 |
| | Compound 1 | −110.2 | O17-O-PHE210, O17-N-GLN289, H1-O-TYR385 | −13.0 |
| Reference Oxaliplatin | | | O14-O-HIS386, O13-O-HIS386, O13-O-ASN382, O13-O-ASN382, N6-O-TYR385 | −21.8 |
| MDM2 (PDB id: 4JRG) | Compound 7 | −92.8 | O18-O-LEU50, O25-O-TYR51, O17-O-VAL89, H64-N-HIS92 | −13.0 |
| | Compound 6 | −104.5 | O23-O-VAL89, O25-O-LYS47, O25-N-TYR51 | −12.0 |
| | Compound 2 | −75.6 | O25-H-LYS47, O18-O-VAL89, O18-N-HIS92 | −10.4 |
| | Compound 4 | −58.5 | O17-O-VAL89, O18-O-LEU50 | −9.0 |
| | Compound 3 | −67.4 | O18-O-LYS47, O22-O-LEU50 | −8.5 |

(Continued)
Hydrophobic interaction analysis showed that all the compounds formed interactions within the active site residues. The residues involved for hydrophobic bonding along with the contributing ligands are described in Supplemental Table S1 and Supplemental Figures S6 to S10. As compound 7 is predicted to be the most potential candidate, a more detailed analysis of the hydrophobic interaction of this molecule with protein models was conducted. For the AKT model, besides hydrogen bond formations, the interaction with compound 7 was further stabilized through hydrophobic bonds with Lys111, Glu114, Glu59, and Asp32. However, the binding site analysis of mToR reveals a total of 10 residues involved in hydrophobic interaction; the residues are Gln1937, Leu1939, Ile1939, Pro2229, Try1974, Ile2228, Ala2226, Glu2196, Gly2142, and Gln2200. In MDM2, key residues involved in hydrophobic interaction; the residues are Gln1937, Leu1939, Ile1939, Pro2229, Try1974, Ile2228, Ala2226, Glu2196, Gly2142, and Gln2200. In MDM2, key residues involved in stabilizing the compound through weak interactions are Tyr61, Gly54, Leu53, Val189, Ile57, Phe87, Ile95, His92, and Tyr96, whereas, at the PDK1 active site, an array of hydrophobic interaction was observed as contributed by Gly89, Glu90, Phe93, Gly91, Ser92, Lys207, Val96, Lys111, and Thr222. In addition, dock pose analysis also indicates the inhibition potential of ligands through the common hydrophobic interaction formed with key residues of studied targets. For example, in the active site of AKT protein binding site, compound 2 was found to exhibit a similar binding pattern to oxaliplatin (Figure 2).

In general, the obtained results suggested that compound 7 may have the potential to simultaneously block AKT, mToR, MDM2, and PDK1, which are critical switches for the hyperactivation of the PI3K pathways as found in many prevalent types of lung cancer. Further, when comparing potent inhibitors for the COX-2 target between compounds 6 and 1, it is found that compound 6 was a better candidate due to its higher number of total hydrogen and hydrophobic interactions formed with this model, which also activates the PI3K pathway through a feedback mechanism. This leads to hypersignaling of AKT functions and in turn cancer relapses. In addition, from the virtual screening scores, compound 2 also exhibited a high docking score to AKT, mToR, and MDM2 targets, which is just slightly lower than that of compounds 6 and 7. Therefore, 4 compounds 1, 2, 6, and 7 are hypothesized to play an important role in targeting cancer therapies with PI3K pathway-related altered signaling.

**Cell Viability Study**

The A549 cell line, human alveolar basal epithelium cells from an explanted culture of lung carcinomatous tissue, was selected as the target for evaluation of the cytotoxicity of the compounds using dimethylthiazol-diphenyltetrazolium bromide (MTT) assay. The control cells showed high proliferation, which could be considered as 100%. Cells were treated with all 7 compounds with various concentrations for 48 h.

From Table 2, the IC50 of compounds 7, 6, 1, and 2 were 11.17 ± 0.8, 12.87 ± 0.3, 18.55 ± 1.3, and 20.07 ± 0.8 µM, respectively, which is higher than that of the reference oxaliplatin, at 22.12 ± 1.1 µM. These initial data demonstrated the capability for proliferation inhibition of A549 cells of enk-kaurane.
Figure 1. Hydrogen bonding interaction: (A) Compound 7, (B) Compound 6, (C) Compound 2, (D) Compound 4, (E) Compound 3, (F) Compound 5, and (G) Compound 1 with the AKT active site residues. Ligands are colored according to element type (CPK coloring) and proteins residues are colored according to amino acid types (Rasmol color scheme).

Abbreviations: CPK, Corey–Pauling–Kuczynski; AKT, Protein Kinase B.
compounds and consistent with results from docking studies, which suggests further investigation into the mechanism of action of these compounds.

Materials and Methods

Protein and Ligand Preparation

Three-dimensional (3D) coordinates for PI3K proteins AKT (PDB id: 1UNQ), mToR (PDB id: 4JSV), COX-2 (PDB id: 3NT1), MDM2 (PDB id: 4JRG), and PDK1 (PDB id: 5LOV) were collected from PDB. The MVD program (6.0, Molegro-a CLC Bio Company) was selected to prepare proteins by assignment of bonds, bond order, charges, and hybridization state. This process removed water molecules and co-factor metal ions followed by adding hydrogen bonds and Tripos atom types in the next step. Piecewise-linear potential (PLP) was adopted for side-chain minimization for hydrogen and steric interactions. Coulomb potential was used for electrostatic forces with constant backbone and varying torsional angles. Cavities were predicted with a probe size of 1.2 Å and a grid size of 0.8 Å.

The 3D structures of small molecules were prepared using MarvinSketch version 19.27.0 and PyMOL version 2.2.2. The energy minimization was carried out using Gabedit 2.5.0.

Docking of Ligands at Active Sites of the Targets and Binding Affinity Calculations

To score each compound, a grid-based scoring system namely MolDock score (GRID) of MVD was adopted. MolDock SE (simplex evolution) was used as search algorithms. The score includes more interaction types like buried, repulsive, nonpolar, hydrogen bonding, and metal, which are scored as follows:

\[
E_{(\text{score})} = E_{(\text{inter})} + E_{(\text{intra})}
\]

\[
E_{(\text{inter})} = \sum_{(\text{ligands})} \sum_{(\text{proteins})} \left[ E_{(\text{PLP})}(r_{ab}) + 332.0 q_a q_b + 4 r_{ab}^2 \right]
\]

where \( E_{(\text{score})} \) is the docking score and \( E_{(\text{inter})} \) is the protein–ligand interaction energy.

The re-rank score for each compound was then used to sort compound poses; the highest scored was considered for evaluating its binding affinity. This process was continued for all the ligands and for all the targets. Binding affinity for the best-ranked pose of each ligand was evaluated in Molegro Data Modeler 3.0 (MDM). Oxaliplatin was considered as a reference for comparing the results.

Hydrophobic Interaction Calculation

Residues involved in hydrophobic interactions were calculated using Ligplot\textsuperscript{+} v2.1 for all the compounds inside the binding pocket along with the reference compound.
Plant Material Preparation

The ent-kaurane diterpenoids were previously isolated from the leaves of the plant C. tonkinensis. The purification and structure identification of the compounds were performed at the Institute of Natural Products Chemistry-Vietnam Academy of Science and Technology. The purity of the compounds was determined as ≥97% by high-performance liquid chromatography. The ent-kaurane diterpenoids and oxaliplatin structures are described in Figure 3.

Cell Line and Cell Culture

The human lung cancer cell line, A549, was obtained from the Center for Nutraceutical and Pharmaceutical Materials, Myongji University. The culture of cells was performed in Dulbecco’s Modified Eagle Medium. The supplementation for the medium consisted of L-alanyl-L-glutamine (2 mM), penicillin (100 g/ml), streptomycin (100 U/ml), and 10% heat-inactivated fetal bovine serum. The atmosphere in which the cells were cultured was humid and had a temperature of 37°C and 5% CO2.

Cell Viability Assay

Upon reaching ~80% confluency, the detachment of cells was performed using 0.05% trypsin/ethylenediamine tetraacetic acid. Cells were then counted, re-suspended, and added onto a 96-well plate, followed by 24 h of attachment. Every tumor cell line was subjected to 48 h of exposure to 7 diterpenoid compounds at 4 concentrations, respectively (100, 10, 5, and 1 μg/ml). The control was selected as oxaliplatin. An MTT assay was adopted to assess cell viability.

Statistical Analysis

At least 3 experimental attempts were performed for all experiments. Results were expressed as mean ± standard deviation. Statistically significant differences were realized at P < .05 via the Student’s t-test.

Conclusions

A total of 7 ent-kaurane diterpenoids from C. tonkinensis were screened in silico for their cancer inhibitory properties through the PI3K signaling pathway targeting AKT, mToR, COX-2, MDM2, and PDK1. Furthermore, all these compounds were tested for their cytotoxic effects against the NSCLC cell line (A549). Compound 7 proved to be the most cytotoxic to the lung cancer cells, followed by compounds 6, 2, and 1 with IC50 lower than that of the reference drug, oxaliplatin. These results are in correlatoin with the conclusion from binding interaction analysis in the molecular docking part. Our work could contribute to the development of targeted anticancer therapy against cancer types where dysregulation of the PI3K pathway occurs like NSCLC and suggests for further in vitro investigation on these compounds with potential for anticancer property.

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Ethical Approval

Ethical Approval is not applicable for this article.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Trial Registration

Not applicable, because this article does not contain any clinical trials.

Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Supplemental Material

Supplemental material for this article is available online.

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