POTENTIAL MORTALIN-p53 COMPLEX ABROGATION OF ENT-KAURANE DITERPENOIDs FROM CROTON TONKINENSIS REVEALED BY HOMOLOGY MODELING AND DOCKING SIMULATION

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Abstract. Seven ent-kaurane diterpenoids from Croton tonkinensis were tested for cytotoxicity against human HCC HepG2 cell line. The abrogation of mortalin-p53 interaction represents an original anticancer therapeutic approach. Tertiary structure of protein mortalin was constructed using Protein Structure Prediction Server and crystal structure of p53 was selected from Protein Data Bank involving mortalin-p53 binding domain. Molecular docking studies revealed that the interaction with protein mortalin was more prominent than p53 and compound 5 and 1 as the most two potential mortalin-p53 binding inhibitors based on binding free energy and interacting residues analysis.

Keywords: Croton tonkinensis, ent-kaurane diterpenoid, cytotoxicity, molecular docking, mortalin-p53.
Classification numbers: 1.2.1, 1.2.4.

1. INTRODUCTION

Croton tonkinensis Gagnep, belongs to Euphorbiaceae family, commonly named in Vietnamese as “Kho sam Bac Bo”, is a tropical shrub native to Northern Viet Nam [1]. It has been used commonly in traditional prescription to treat leprosy, psoriasis, malaria and genital organ prolapse [1, 2]. Ent-kaurane-type diterpenoids from Croton tonkinensis have been well known for cytotoxic properties against several cancer cell lines such as: breast (MCF-7), lung (A549), colon (LS180) [3 - 5]. However, cytotoxicity on human HCC cell line was not investigated.

Among the innovative approaches developed in the past decade in drug discovery, targeting protein-protein interactions has emerged as a potent strategy in oncology [6]. In this field, efforts have been made to search for small molecules with potential to inhibit the interaction between
p53 and proteins which negatively inactivated the tumor suppression functions of p53. Promising results have been obtained with inhibitors of the interaction between p53 and the ubiquitin ligase MDM2 which are currently tested in phase I trials [7]. Mortalin is a stress chaperone of Hsp70 family of proteins that performs various functions related to proliferation, mitochondrial biogenesis, chaperoning and stress response [8, 9]. In the previous studies, it has been demonstrated that mortalin associates with liver cancer metastasis and can be used as a marker to predict early tumor recurrence [10]. Mortalin binds to p53 tumor suppressor protein and sequesters it in the cytoplasm, resulting in an inhibition of the transcriptional activation and control of centrosome duplication functions of p53, thus causing lifespan extension of normal human cells and increase malignant properties of human cancer cells [11 - 13]. It is expected that the abrogation of mortalin-p53 interaction will reactivate p53 function. This could represent an original anticancer therapeutic approach.

In this study, we conducted cytotoxicity assay of 7 ent-kaurane diterpenoids from Croton tonkinensis against HepG2 cancer cell line. Homology modeling was used to determine the 3D structure of mortalin and p53 crystal structure involved mortalin-binding site was selected. All studied compounds were submitted for molecular docking to investigate inhibition mechanism of bioactive compounds.

2. MATERIALS AND METHODS

2.1. Tested compounds

Compounds 1-7 were provided by Prof. Pham Thi Hong Minh - Institute of Natural Products Chemistry, Vietnam Academy of Science and Technology. All compounds achieved the purity of ≥ 97 % as determined by HPLC. The ent-kaurane diterpenoids and solasonine structures are described in Figure 1.

![Figure 1](image-url)

*Figure 1.* (A) The chemical structures of ent-kaurane diterpenoids 1 to 7, the chemical entities of groups R₁ to R₅ for each compound (1 to 7) are specified. (B) Chemical structure of solasonine.
2.2. Cell culture and cell viability assay

The human liver cancer cell line HepG2 were originally from the ATCC. Cells were grown in monolayer using Dulbecco’s modified Eagle’s medium (DMEM) (Hyclone, USA) supplemented with 10 % fetal bovine serum (Hyclone), penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37 °C in a humidified atmosphere with 5 % CO₂ and 95 % humidity. Cell viability was assessed based on the MTT protocol described by Mosmann [14].

2.3. Homology modeling and protein preparation

The tetramerization domain crystal structure of p53 protein was obtained from Protein Data Bank (PDB ID: 1AIE) encompassing the mortalin-binding site (residue 326-356) [15]. The tertiary structure of protein mortalin is not well determined in previous studies, thus, the structure was constructed by comparative modeling using MODELLER package from the Protein Structure Prediction Server (PS²-v3 server) [16]. The amino acid sequences of mortalin (Accession ID: P38646) were obtained from UniProt website (Table 1) which consist of p53-binding site in the peptide binding domain. The predicted 3D structure was validated using PROCHECK to evaluate backbone conformation based on Psi/Phi Ramachandran plot analysis.

2.4. Ligand preparation

MarvinSketch 19.27.0 was used to draw structure of ent-kaurane diterpenoids. Solasonine was proved to inhibit the mortalin-p53 interaction, thus, selected as standard inhibitor. The 3D conformation of these compounds were built using PyMol 2.2.2 [17]. The energy minimization was carried out using Gabledit 2.5.0 and Chemicalize webserver prior docking [18].

2.5. Molecular docking study

Proteins and ligands were prepared for docking using AutoDock Tools 1.5.7 (ADT). The heteroatoms including water molecules were deleted and polar hydrogen atoms and Kollman charges were added to the receptor molecule. ADT assigns the rigid roots to the ligand automatically, all other bonds were allowed to be rotatable. It was reported in previous studies that the p53-binding site of mortalin resides in the peptide binding domain (residues 439-597) [19, 20], therefore, the location and dimensions of the grid box for each protein was chosen such that it incorporates the amino acids domain involved in the mortalin-p53 binding site which was enclosed in a box with the number of grid points in x × y × z directions and a grid spacing of 0.375 Å. AutoDock 4.2.6 was utilized for the molecular docking simulation. All the docking simulations were performed in Intel®Core™ i7-9700K CPU @ 3.60 GHz, with 32 GB DDR4 RAM.

3. RESULTS AND DISCUSSION

3.1. Cell viability assay

All the studied compounds were evaluated for cytotoxicity on HepG2 cell line (Table 1). The obtained results indicated that amongst 7 ent-kaurane diterpenoids, compound 3 exhibited the highest IC₅₀ value on HepG2 (85.2 μM). Compound 5 was the most active with IC₅₀ value around 4.6 μM, followed by compound 1 and 4 (5.1 μM and 5.9 μM, respectively). To a lesser
extent, compound 6 and 2 displayed similar cytotoxic activities with IC$_{50}$ value close to 10 μM. It should be noted that, all the compounds except compound 3 have the O=C-CH=CH$_2$ system, thus, suggest the 16-en-15-one basic skeleton plays an important role in the cytotoxic activities of these diterpenoids.

Table 1. Cytotoxicity activity (IC$_{50}$, μM) of ent-kaurane diterpenoids 1 to 7 on human HCC HepG2 cell line after 48 hours of incubation.

| Compound | IC$_{50}$ (μM) | Compound | IC$_{50}$ (μM) |
|----------|----------------|----------|----------------|
| 1        | 5.1 ± 1.5      | 5        | 4.6 ± 0.8      |
| 2        | 9.8 ± 3.1      | 6        | 8.0 ± 1.2      |
| 3        | 85.2 ± 32.5    | 7        | 13.7 ± 1.3     |
| 4        | 5.9 ± 0.3      | Solasonine | 4.5 ± 0.2     |

3.2. Homology modeling and protein preparation

Tertiary structure of drug target is the initial requirement for structure-based drug design. In the absence of an experimentally determined structure, homology modeling is an efficient method for 3D structure prediction and quick experimental design for docking studies. In general, the target sequence should have at least 30 % sequence identity with an experimentally determined structure for generating useful 3D models. Crystal structure of the molecular chaperone DnaK from *Geobacillus kaustophilus* HTA426 in post-ATP hydrolysis state (PDB ID: 2v7y) showed highest sequence identity (64.96 %) with the drug target, hence, was selected as the template (Figure 2).

Figure 2. Sequence alignment of target (mortalin) and template protein (PDB ID: 2v7y).
In general, eight homology models of mortalin were generated. Conformational energy represents the stability of a conformation with respect to other conformations of the same protein. Measure of conformational energy is represented as DOPE score in homology modeling. Lower DOPE score represents relatively more stable 3D conformation of the drug target. In this study, the third model of mortalin with lowest DOPE score was selected for further docking simulation (Figure 3A). The Ramachandran plot showed 93% residues in favorable regions (Figure 3B). Evaluation of the predicted model using ProSA has revealed that the Z-score value (-5.21) is well within the range of native conformations of crystal structure of similar length (Figure 3C). The overall residue energies were mainly negative which suggest the good quality of the mortalin model (Figure 3D).

Figure 3. (A) Validation of predicted mortalin model based on DOPE score; (B) PROCHECK evaluation; (C) ProSA evaluation: Z-score plot; (D) ProSA evaluation: energy plot.

In summary, two protein models of mortalin and p53 (Figure 4) consist of key residues in the mortalin-p53 binding site have been constructed and prepared for docking simulation.

3.3. Docking studies

Table 2 displayed the binding affinity of 7 ent-kaurane diterpenoids toward targeted proteins and key residues involved in forming interaction. Solasonine was used as standard ligand for docking validation. According to the ranking criteria of Autodock 4.2.6, the more negative value of docking energy, the better binding affinity of the compound towards targeted
receptor. The obtained results showed high correlation between binding affinity and the cytotoxic activities, which suggest a hypothesis that compounds with better binding affinity will likely to exhibit more toxicity against HepG2 cell line.

![Figure 4](image)

Figure 4. Protein model of mortalin (A) and p53 (B).

Table 2. Docking score and interacting residue of ent-kaurane diterpenoids with mortalin and p53.

| Compounds | 3N8E (Mortalin) | 1A1E (p53) |
|-----------|----------------|------------|
|           | Binding free energy (kcal/mol) | No. of H-bond | Interacting amino acids | Binding free energy (kcal/mol) | No. of H-bond | Interacting amino acids |
| 1         | -15.33         | 4          | Ala475, Arg513, Glu586, Ser473, Gln479 | -11.42 | 2 | Leu330 |
| 2         | -12.88         | 2          | Leu450, Ser473 | -9.52 | 1 | Leu330 |
| 3         | -8.04          | 2          | Leu450, Ser473 | -8.32 | 2 | Leu330, Arg342, Asn345, Glu349 |
| 4         | -14.41         | 1          | Glu483 | -12.73 | 3 | Leu330, Arg342, Asn345, Glu349 |
| 5         | -15.78         | 5          | Ala475, Arg513, Glu586 | -13.06 | 2 | Phe328 |
| 6         | -13.98         | 1          | Thr455 | -10.40 | 1 | Thr329 |
| 7         | -11.85         | 2          | Thr455, Asn583 | -10.45 | 2 | Asn345 |
| Solasonine | -15.62         | 4          | Ala475, Arg513, Glu577, Glu586 | -14.53 | 3 | Glu326, Phe328, Asp352 |

As reported in previous studies, the key residues involved in forming mortalin-p53 interaction were Ala475, Arg513, Glu586 for mortalin and Glu326, Phe328 and Asp352 for p53 [20, 21]. In this research, solasonine, the standard mortalin-p53 interaction inhibitor, was observed to form 4 hydrogen bonds with mortalin model through Ala475, Arg513, Glu577 and Glu586 meanwhile, the formation of H-bonds with Glu326, Phe328 and Asp352 is essential for binding with p53 (Figure 5). In addition, solasonine tends to interact with mortalin more efficiently than p53 due to more negative dock score (-15.62 and -14.53 kcal/mol).
In general, docking analysis showed that most of the diterpenoids except compound 3 are more likely to interact with mortalin than p53 protein. Compounds 5 and 1 were indicated as the most potential inhibitors based on binding free energy criteria (-15.78 and -13.06 kcal/mol). Interestingly, all studied compounds do not form interaction with key residues in p53 protein (Table 2). This observation suggests that these diterpenoids were not favored to inhibit p53 in the mortalin-binding site. On the other hand, for mortalin model, only compound 5 and 1 share common H-bonds residue with solasonine. These two compounds docked into p53-binding site of mortalin through hydrogen bonds with Ala475, Arg513, Glu586 (Figure 5). Obtained results

Figure 5. Hydrogen bonding patterns of ent-kaurane diterpenoids with mortalin (3N8E) and p53 (1AIE). (A) I docked with mortalin; (B) I docked with p53; (C) S docked with mortalin; (D) S docked with p53; (E) Solasonine docked with mortalin; (F) Solasonine docked with p53.
reveal that compounds 5 and 1 could be considered as potential mortalin-p53 inhibitors meanwhile the mechanism of action of the other compounds are yet to be explore.

4. CONCLUSIONS

In this study, 7 ent-kaurane diterpenoids from *Croton tonkinensis* have been tested for cytotoxicity assay on HepG2 cell line. Most of the compounds exhibited bioactivities except compound 3, compounds 5 and 1 were indicated as the most toxic against HepG2. Since the crystal structure of mortalin has not been clearly determined, the tertiary structure of mortalin was constructed using homology modeling. Docking studies revealed compounds 5 and 1 as the most potential to inhibit the interaction between mortalin and p53 based on high binding affinity and hydrogen bonds formed with key residues.

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Declaration of competing interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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