Molecular docking and molecular dynamic study of multiple medicinal plants' bioactive compounds as human papillomavirus type 16 E5 protein inhibitor

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Abstract

Cervical cancer is the second most prevalent form of cancer in Indonesia. HPV16 and HPV 18 are the leading causes of cervical cancer, accounting for 70-90% of cases. The E5 protein may play a critical role in the disease's development. Although the high-risk (HR) version of this protein may have some benefits in evading the immune system through MHC I and influencing the cell cycle via p21/p27, very few studies have been performed owing to its tiny size and high hydrophobicity. The purpose of this research is to predict the anti-viral activity of asarinin and thiazolo[3,2-a]benzimidazole-3(2H)-one,2-(2-fluorobenzylideno)-7,8-dimethyl (thiazolo) using molecular docking and molecular dynamics. The docking results showed that the two candidate drugs had a lower docking affinity than rimantadine but comparable stability. Both potent compounds are predicted to disrupt MHC I localization in the ER, the ability of infected cells to proliferate, and the virion assembly process. In contrast, rimantadine is predicted to disrupt infected cells' proliferation ability via the epidermal growth factor receptor (EGFR) regulation and inhibit the activation process of mitogenic signalling in keratinocytes.

Keywords: Cervical Cancer, dynamic simulation, E5 protein, HPV16, molecular docking.

1. Introduction

Cervical cancer is the second most prevalent form of cancer in Indonesia and the second most common type of cancer in females aged 15 to 44 years (Domingo *et al.*, 2008; Nurcahyanti, 2016). Cervical cancer accounted for approximately 9-10% of all cancer cases reported in 2018 (Arbyn *et al.*, 2020). Every year, its incidence and prevalence rates tend to increase by around 17 and 20%, respectively (Wahidin *et al.*, 2020). Primarily, cervical cancer is caused by Human Papillomavirus (HPV), a collection of non-enveloped DNA viruses that primarily infect the keratinocytes' basal layer. It is known to be spread by skin-to-skin contact, most notably through sexual intercourse (Tao *et al.*, 2003). There are now over 200 different kinds of HPV, which are usually classified into five genotype groups and two risk categories, high-risk (HR) and low-risk (LR) HPV (Bzhalava *et al.*, 2013; Venuti *et al.*, 2011). HPV16 and 18 are the leading causes of anogenital malignancies, most commonly cervical cancer, and are the leading causes of concern for HPVs. Around 70–90% of cervical cancer cases are caused by HPV16 and HPV 18, respectively, with HPV16 alone accounting for 55% of all instances (Graham, 2017; Venuti *et al.*, 2011; Wang *et al.*, 2018). Around 4% of women in the general population are thought to be exposed to HPV16 or 18 at any particular time (ICO/IARC HPV Information Centre, 2019). Additionally, high-risk HPV might have a crucial role in the pathogenesis of various forms of cancer, such as the respiratory tract, eyes, esophagus, non-small-cell lung, colorectal, breast, prostatic, and urinary bladder cancers (Venuti *et al.*, 2011).

The majority of research on HPV-related cancer has concentrated on two oncoproteins, the E6 and E7 proteins (Doorbar *et al.*, 2012; Pal & Kundu, 2020; Yeo-Teh *et al.*, 2018). These proteins are essential oncoproteins that distinguish high-risk from low-risk variants, owing to their well-characterized actions and pathways (Graham, 2017). For example, E6 interacts with and degrades the cellular tumor suppressor p53, while E7 interacts with and inactivates the retinoblastoma (Rb) proteins, resulting in tumor development. (Doorbar *et al.*, 2012; Egawa & Doorbar, 2017; Tao *et al.*, 2003; Underbrink *et al.*, 2016). Also, both proteins interact with various proteins that leads mainly to immune evasion and genomic instability (Yeo-Teh *et al.*, 2018). Some research suggests another significant oncoprotein in the HR type of HPV is the E5 protein, an 83 amino acid long hydrophobic transmembrane protein that interacts with various cellular proteins and required for the protein's biological activity during cell transformation (Campo *et al.*, 2010; DiMaio & Petti, 2013; Krawczyk *et al.*, 2010; Suprynowicz *et al.*, 2008). Some effects caused by E5 protein activity are relocalization of calpactin I to the perinuclear region, enhancement of growth factor signaling patterns by activation of EGF-R, suppressing three key proteins of the ER stress pathway such as cyclooxygenase-2 (COX-2), XBP-1, and IRE1a, and downregulation of the Major Histocompatibility Complex class (MHC I) (DiMaio & Petti, 2013; Gruener *et al.*, 2007; Venuti *et al.*, 2011). In addition, E5 also alters endosomal pH by interacting with the vacuolar H+-ATPase, acidifying cellular organelles. However, due to their tiny size and hydrophobicity, the E5 proteins lack the substantial soluble, globular domains required for specialized protein-protein interactions (DiMaio & Petti, 2013; Disbrow *et al.*, 2005; Marshansky & Futai, 2008; Venuti *et al.*, 2011). Additionally, HR HPV E5 is engaged in the early stages of carcinogenesis by extending the survival of infected cells and increasing their pool. However, the E5 ORF is almost exclusively found in the HR-HPV genome since it is missing from the genomes of many other HPVs, including beta-, gamma-,

and mu-HPVs, suggesting that the protein is not required for the virus's life cycle but may provide some advantage on infection and transformation (Doorbar *et al.*, 2012; Longworth & Laimins, 2004; Wang *et al.*, 2018).

As one of the largest tropical countries, Indonesia is home to about 7000 medicinal plants. However, less than 10% of these taxa are classified as phytopharmaca (Salim & Munadi, 2017). For hundreds of years, the Indonesian culture has depended on a range of medicinal plants to treat or alleviate mild to severe diseases, backed up by empirical evidence from the community, even though scientific data is often sparse. Around 55% of Indonesians regularly use alternative treatments, and over 95% think they benefit from them (Jennifer & Saptutyningsih, 2015; Sumayyah & Salsabila, 2017). These results lay the groundwork for future research into treatment options for various diseases, including HR-HPV infection. Research into drug creation would be a lengthy endeavor, much more so if the components are natural. Chemical screening, as well as screening and testing *in silico*, is one of the first stages, as shown in this study (Kitchen *et al.*, 2004; Lionta *et al.*, 2014). Our previous study in HPV16 E6 protein showed that the two most potent compounds against E6 protein are asarinin and thiazolo, found in *Zanthoxylum spp* and *Myristica fragrans,* respectively. This study aimed to target the HPV16 E5 protein directly with those specific compounds, hoping for potent inhibition activity in the protein target activities.

2. Materials and Methods

The objective of this research was to target the HPV16 E5 oncoprotein specifically. The amino acid sequence for the target protein was acquired from UniProt (https://www.uniprot.org/uniprot) under the accession number P06927. The 3D structure was then modeled using I-TASSER webserver (https://zhanglab.dcmb.med.umich.edu/I-TASSER/). The modeled was chosen based on the C-score value and RMSD score, representing suitable position of each atom to be modelled.

Asarinin (CID: 11869417) and thiazolo (CID: 1823738) are used as particularly potent natural compounds, whereas Rimantadine (CID: 5071) is used as a control (Wetherill *et al.*, 2012). All of these possible chemicals were obtained in SDF format from PubChem (https://pubchem.ncbi.nlm.nih.gov/).

2.1. Pathway Analysis

The HPV infection pathway analysis in the host cells is based on KEGG's database (https://www.genome.jp/kegg/) to identify which protein and biological process disrupted by HPV16 E5 protein inducing the cancer development. Through the KEGG map, then we analyzed the potential target protein related to the HPV infection.

2.2. Molecular Docking Process

AutoDock Vina is used for docking, which is integrated with PyRx (https://pyrx.sourceforge.io/) (Trott & Olson, 2009). First, we examine the target protein's complete structure. The molecular coverage area (in Angstroms) is 39.3567; 40.9481; 35.1306, whereas the center coordinates are 56.3238; 40.9481; 35.1306, respectively. The primary docking findings are the compound's

affinity in kcal/mol, the location of the binding site, and the subsequent visualization of the protein-ligand interaction.

2.3. Visualization Process

The visualization method is divided into two stages: the 3D visualization of possible chemical binding sites on the E5 protein and the 2D visualization of interaction in each protein-ligand combination. PyMOL (https://pymol.org/2/) is used for 3D visualization, whereas LigPlot+ 2.1 (https://www.ebi.ac.uk/thornton-srv/software/LigPlus/) is used for 2D visualization.

2.4. Molecular Dynamics Simulations

Ligands with the lowest binding affinity scores were selected for molecular dynamic simulation against E5 protein. The parameters were set up according to the normal physiological conditions (37°C, 1 atm, pH 7.4, 0.9% salt content) for 1000 picoseconds simulation time. Molecular dynamics simulation was run through md_run macro program, and the analysis followed by using md_analyze and md_analyeres on yasara program.

3. Results

3.1. Pathway Analysis Results

The infection pathway in KEGG (hsa05165) revealed that E5 protein activity was associated with three distinct biological processes: immune evasion via inhibition of MHC I in the endoplasmic reticulum (ER), activation of a growth factor such as platelet-derived growth factor receptor beta (PDGFRB) that directly stimulates cell proliferation, and immortalization mechanisms via MAPK and calcium signaling pathways.

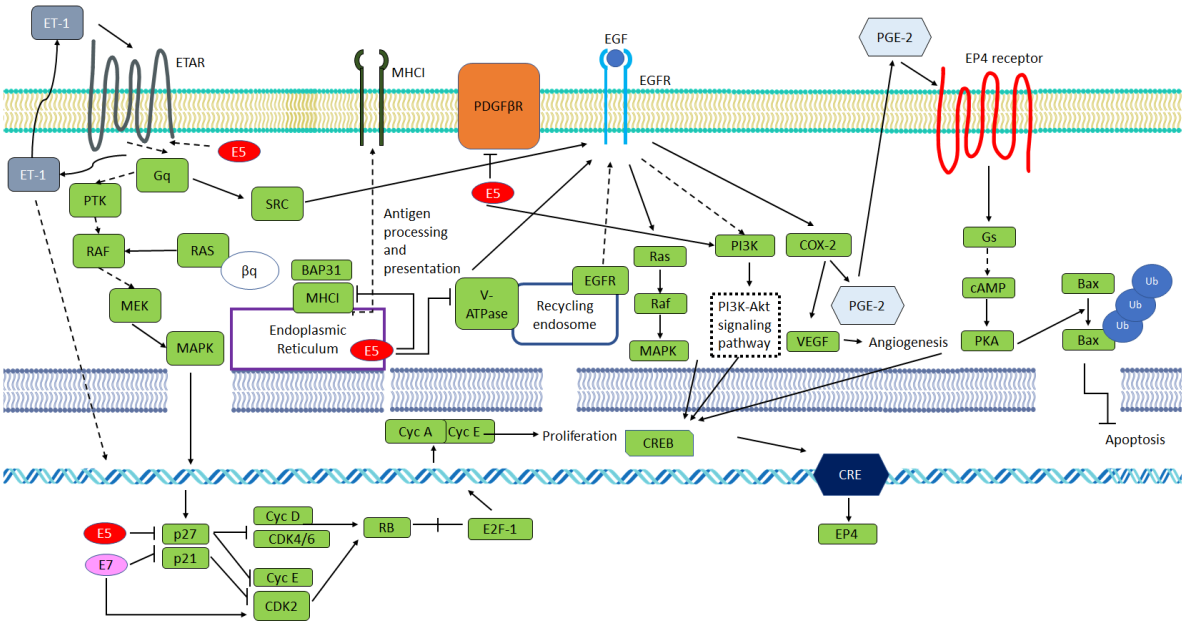

Fig. 1. The infection pathway of HPV in KEGG (hsa05165). A showed E5 protein promotes PDGFRB activation and immune evasion mechanism by downregulating the MHC I. B showed E5 protein bypassing infected cell straight to S-phase by inhibiting p21 and p27.

In figure 1A, it appears as though the HPV16 E5 protein localized to the ER membrane is intending to suppress adaptive immune responses by downregulating MHC class I, thereby presenting ER-derived peptides on the cell surface for exposure to cytotoxic T- cells that cannot accumulate on the surface of infected cells. Additionally, it appears as though E5 protein interacts with B-cell receptor-associated protein 31 (BAP31). BAP31 is a ubiquitously expressed transmembrane protein found primarily in the ER that acts as a chaperone protein for MHCI, forcing it to localize to the ER and Golgi and maintain an infected host cell proliferation-competent state (Dang *et al.*, 2018; Quistgaard, 2021; Regan & Laimins, 2008).

Additionally, the KEGG pathway showed that the E5 protein suppresses tumor suppressors p21 and p27 (figure 1B), which are cell cycle inhibitors that induce cells to enter the S-phase by inhibiting G1-phase markers such as CDK2/4/6 and Cys D/E, thus disrupting cell cycle checkpoint mechanisms. In addition, it causes infected cells aggressively synthesize DNA during the S phase, resulting in the formation of koilocytes, which are often employed as a morphological marker of HR-HPV infection. The KEGG viral carcinogenesis pathway (not shown here) also reveals that the HPV16 E5 protein downregulates the V-ATPase protein, particularly the 16 kDa subunit of V-ATPase disrupts endosomal acidification, resulting in increased EFGR recycling and active cell proliferation. Because all biological processes affected by the E5 protein contribute to cancer development, the HPV16 E5 protein was classified as an oncoprotein in this research.

3.2. Molecular Docking Results

The docking results (Table 1) show that the two potential natural compounds exhibited 50% and 31% lower affinity values than the control drug (rimantadine; -4.8 kcal/mol). As a result, these compounds should have a higher propensity for initiating interactions with the target protein than drug controls (Murcko & Ajay, 1995). The negative value in the results represents the spontaneous interaction between the ligand and target protein when the ligand-protein complex reaches equilibrium under constant pressure and temperature conditions simulated throughout the docking process (Bronowska, 2011; Du *et al.*, 2016; Murcko & Ajay, 1995).

Table 1. Docking and 2D visualization two potent compounds against HPV16 E5 protein

Compounds	ΔG	Amino Acid Residue	Interactions (Å)
Asarinin (CID: 11869417) *Zanthoxylum spp* (bark)	-7.2 (Kcal/mol)	Thr76; Leu71; Ser37; Tyr39; Ser35; Leu23; Ala78; Pro31	Hydrophobic contact
		Arg79	Hydrophobic contact
			Hydrogen bond (2.92)
Thiazolo[3,2-a]benzimidazol-3(2H)-one,2-(2-fluorobenzylideno)-7,8-dimethyl (CID: 1823738) *Myristica fragrans* (seeds)	-6.3 (Kcal/mol)	Tyr39; Leu23; Thr76; Ser35; Ala78; Leu71	Hydrophobic contact
Rimantadine (CID: 5071) *Drug controls*	-4.8 (Kcal/mol)	Pro70; Thr38; Leu71; Ile64; Ser41; Ile43	Hydrophobic contact
		Tyr39	Hydrophobic contact
			Hydrogen bond (2.97)

3.3. Post-docking Visualization Results

The results of the post-docking visualization results demonstrate that asarinin and thiazolo did not intersect at the same binding site as the control. However, when verified using two-dimensional visualization, two residues, Tyr39 and Leu71, were shown to be shared by the control and potent compounds (figure 2).

Additionally, 2D visualization demonstrates that asarinin and thiazolo are colocalized at the same binding site, flanking the first hydrophobic domain (first α-helix; red), the third hydrophobic domain (third α-helix; yellow), and the C-terminal region (cyan). Thus, Asarinin interacts with the first hydrophobic domains located at Leu23 and Pro31, the third hydrophobic domain located at Leu71, and the C-terminal at Thr76, Ala78, and Arg79. Thiazolo binds to the first hydrophobic domain at Leu23, the third hydrophobic domain at Leu71, and the C-terminal area at Thr76, Ala78. These findings show that the two natural chemicals (Leu23, Thr76, and Leu71) share conserved residues, indicating that the two possible compounds bind to the same binding site. Simultaneously, it was anticipated that rimantadine, as a control, would interact with the second and third hydrophobic domains at Phe43, Ile64, Pro70, and Leu71.

3.4. Molecular Dynamic Simulation Results

To assess docked complexes' flexibility and overall stability, we performed a time-dependent MD simulation at 1000 picoseconds. The energy potential graph reveals that all E5 complexes have near identical values and a horizontal trend throughout the simulation period, about -4.96e5 kJ/mol, indicating that all complexes were energetically stable during the simulation (figure 3).

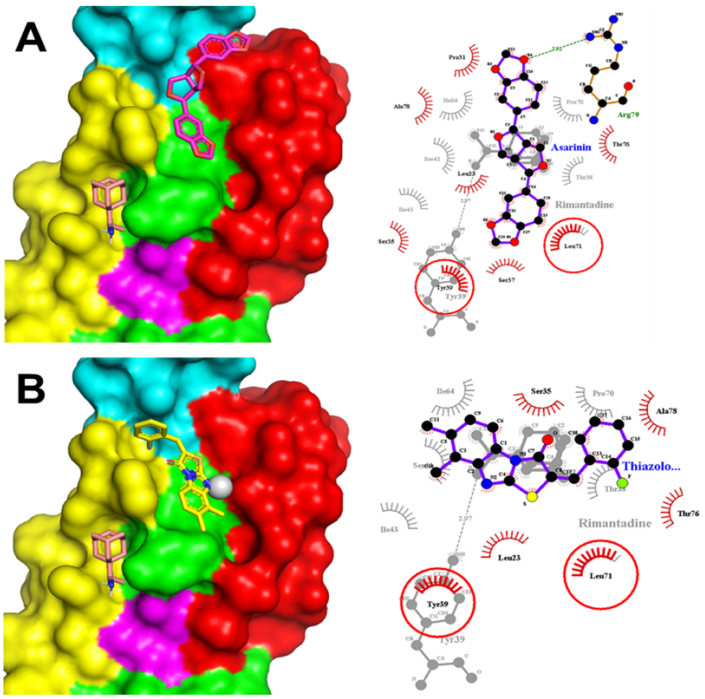

Fig. 2. An overview of the binding sites based on 3D visualization results with HPV16 E5 protein. The interaction between the two potent compounds with HPV16 E5 protein is based on the 2D visualization results. (A) asarinin, (B) thiazolo[3,2-a]benzimidazol-3(2H)-one. First hydrophobic domain (first α-helix; red), the third hydrophobic domain (third α-helix; yellow), and the C-terminal region (cyan).

The root mean square deviation was determined throughout the course of 1000 picoseconds of simulation (figure 4). The RMSD results of E5-asarinin showed a mean value of 5.915±1.099Å with a minimum value of 1.166Å and a maximum value of 7.316Å. The E5-thiazolo complex showed a mean value of 5.067±0.733Å with a minimum value of 1.231Å and a maximum value of 6.062. The E5-rimantadine showed a mean value of 5.396±1.176Å with a minimum value of 1.181Å and a maximum value of 7.113Å.

The RMSD values indicated that the E5-thiazolo complex exhibited minor variations at 100, 350, and 950 ps, but stabilized in about 5Å after 150 ps. Meanwhile, the E5-asarinin complex stabilized after 150 ps but deviated to approximately 7Å at 550 ps and subsequently exhibited a decreasing trend in the RMSD value after 600 ps until the conclusion of the test period. Before 550 ps, the E5-rimantadine complex exhibited a constant RMSD value less than 6Å, but indicating an increasing trend in value from that point until the simulation period ended. Stable RMSD values suggest that the interactions established in the complex are stable, implying that the target protein will tend to retain its structure, while variations in the RMSD value are due to changes in the conformation of the chemical tested at its binding site.

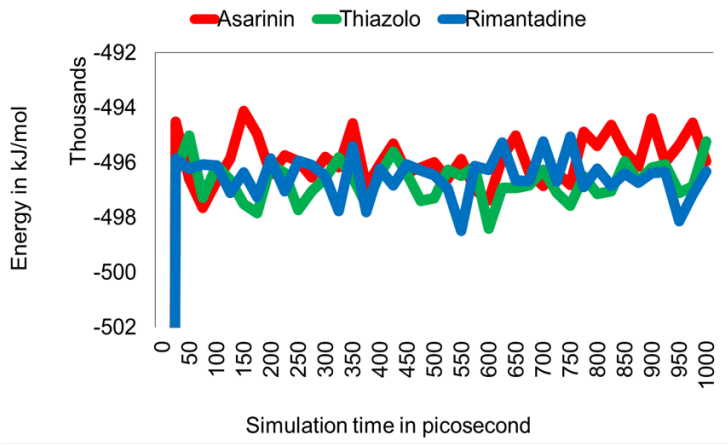

Fig. 3. Total potential energy of the system among HPV16 E5 and ligands interaction over a 1000 picosecond simulation

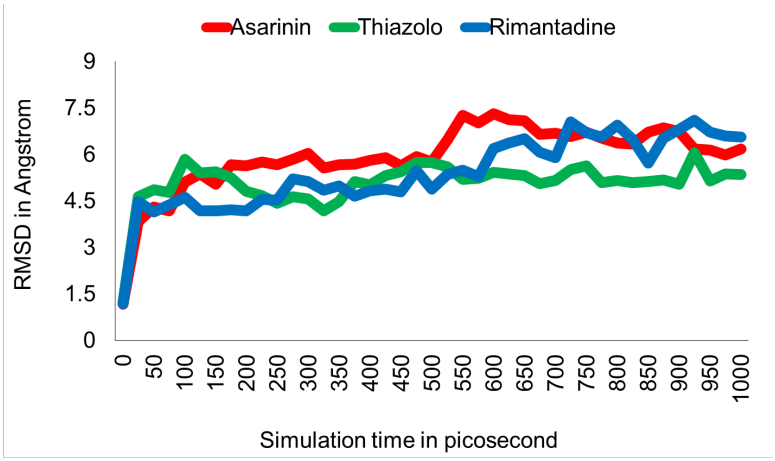

Fig. 4. RMSD plot showed the stability of protein-ligand complex interaction among HPV16 E5 and ligands over a 1000 picosecond simulation

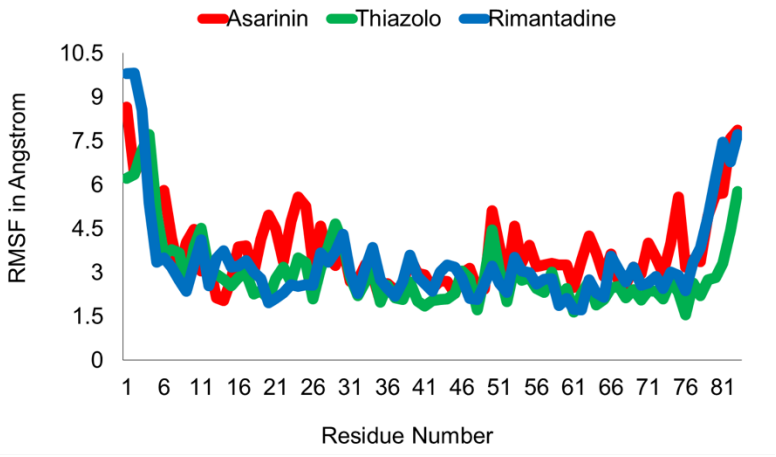

Fig. 5. RMSF plot showed the stability of amino acid residues among HPV16 E5 and ligands over a 1000 picosecond simulation

The RMSF plot (figure 5) demonstrates that no major differences occurred between the three tested substances throughout the simulation period. The E5-thiazolo, and E5-rimantadine complexes, on the other hand, consistently demonstrated lower RMSF values than the E5-asarinin complex. Because the RMSF value did not fluctuate much in any of the three complexes examined, it was believed that the three protein-ligand complexes produced were in a stable conformation. However, the data reveals that asarinin had the greatest variability, even more than the other two chemicals examined. The RMSF figure reveals that the least flexible amino acid residues are clustered around the second hydrophobic domain (AA 36-50). Except at the extreme C-terminus, the RMSF value of the residue that interacted with each compound was often lower than that of the other residues in each complex examined.

4. Discussion

The HPV16 E5 protein is a critical component of the HPV infection mechanism. Along with the E6 and E7 proteins, the much smaller E5 protein is believed to be a critical oncoprotein, particularly during cancer development (DiMaio & Petti, 2013; Venuti *et al.*, 2011; Wetherill *et al.*, 2018). Due to the protein's small size and highly hydrophobic structure, few studies have examined it in detail (Wetherill *et al.*, 2012; Nath *et al.*, 2006). According to the KEGG pathway, the HPV16 E5 protein plays several critical roles, including immune evasion by downregulating MHC I and BAP31 in the ER and promoting infected cells to S-phase by inhibiting the tumor suppressors' p21 and p27. Due to the breadth of its functions, despite its small size and relatively simple structure, HPV16 E5 contains a large number of active domains, including the first hydrophobic domain/first helix (AA 11-34) that interacts with a heavy chain of MHC I via leucine pairs, resulting in the downregulation of MHC I, the five final AA in the C-terminus that are thought to interact with the epidermal growth factor receptor (EGFR), and the third hydrophobic domain/first α-helix (Ashrafi *et al.*, 2006; Wetherill *et al.*, 2012; Nath *et al.*, 2006; Regan & Laimins, 2008; Rodríguez *et al.*, 2000; Venuti *et al.*, 2011).

The docking findings indicated that the two potential compounds previously evaluated on the HPV16 E5 protein had a lower docking affinity value than rimantadine, implying a greater and more stable probability of contact between the two potent compounds and the E5

protein (Murcko & Ajay, 1995). However, when the RMSD and RMSF values were compared, only thiazolo had a lower mean value than rimantadine. The potential energy of the three complexes was investigated, and it was observed that they remained constant during the simulation duration. It suggests that the electrostatic and van der Waals interactions of the three complexes remained constant during simulation, implying that no abnormalities occurred during the process (Albaugh *et al.*, 2016). The RMSD plot revealed that thiazolo exhibited the most stable interaction of the three compounds complex examined, but its docking affinity was lower than asarinin. Meanwhile, asarinin, which has the highest docking affinity, exhibits similar stability to rimantadine, if not slightly worse, as it exhibits a slightly higher flexibility pattern than control, indicating that the E5-asarinin complex is relatively less stable when compared to control and thiazolo (Aier *et al.*, 2016; Schneider *et al.*, 2011; Sivaramakrishnan *et al.*, 2020).

The two possible compounds are expected to interact with three key regions of the E5 protein, particularly the first hydrophobic domain, the third hydrophobic domain, and the C-terminus, based on the findings of the 2D visualization and the RMSF plot. Stabilized interaction with the first hydrophobic domain, particularly with Leu23 and Pro31, which are located in the middle of the α-helix structure, is thought to disrupt the E5 protein's interaction with the heavy chain of MHC I, resulting in delocalization of MHC I in the ER, thereby destroying the virus's immune evasion mechanism (Abe *et al.*, 2009; Campo *et al.*, 2010; Gruener *et al.*, 2007; Nath *et al.*, 2006; Venuti *et al.*, 2011). Both compounds are also believed to disrupt the endosomal acidification process mediated by v-ATPase, thus decreasing the rate of EFG-receptor recycling to the plasma membrane. Consequently, these infected cells lack the number of EFGR receptors seen in other infected cells and do not achieve the EFGR ratio found in normal cells (Ilahi & Bhatti, 2020; Müller *et al.*, 2015; Venuti *et al.*, 2011). Although a decreased EFGR ratio is anticipated to delay the proliferation and angiogenesis of infected cells, the percentage of reduction cannot be predicted. Potent drugs interacting with extreme C-terminus residues such as Thr76 and Ala78 would inhibit future EGFR pathway hyperactivation, eventually reducing the cell's proliferative capacity. Additionally, it is believed that potent compounds interacting with the extreme C-terminus inhibit the interaction of E5 protein with BAP31, which is involved in the export of MHC I to the cell surface, the maintenance of infected cells' proliferation ability, and the virion assembly process, resulting in decreased expression in infected cells' MHC I is in its membrane, its proliferation is less aggressive than other infected cells, and the rate of HPV virion assembly is also lower (Abe *et al.*, 2009; DiMaio & Petti, 2013; Ilahi & Bhatti, 2020; Kotnik Halavaty *et al.*, 2014; Müller *et al.*, 2015; Regan & Laimins, 2008).

Rimantadine demonstrated a distinct inhibition pattern of the E5 protein compared to the other two natural drugs examined, based on docking and visualization results. Rimantadine is expected to interact with only third hydrophobic domains through Pro70, Leu71, and Ile64, resulting in a decrease in endosomal acidification and the rate of EGFR recycling. Iso64 and Pro70 are also suspected of functioning as voltage gating motifs, with interactions between these residues potentially inhibiting the diffusion of certain critical ions, although the intricacies of the voltage gating motif's function on the HPV16 E5 protein remain mostly unclear (Nath *et al.*, 2006; Scott & Griffin, 2015). More often referred to as an anti-viroporin, rimantadine is believed to bind with one of the residues that comprise the viroporin's lumen in its oligomer

state structure, such as Ser41 and therefore block the activation process of mitogenic signaling in keratinocytes. A comparable direct blockage of the viroporin channel is believed to be caused by the interaction between Ser37, which constitutes the viroporin lumen, and asarinin (Wetherill *et al.*, 2012; Scott & Griffin, 2015; Wetherill *et al.*, 2018).

5. Conclusion

The HPV16 E5 protein plays a vital role in the HPV infection process. By suppressing the tumor suppressors p21 and p27, it downregulates MHC I and BAP31 in the ER and promotes the entry of infected cells into the S-phase. The docking results indicated that asarinin and thiazolo had a lower docking affinity value than rimantadine, implying a greater and more stable probability of contact between the two compounds and the protein, but comparable stability as measured by the RMSD and RMSF plots. Both potent compounds are predicted to disrupt MHC I localization in the ER, infected cells' ability to proliferate, and the virion assembly process. In contrast, rimantadine is expected to impair infected cells' proliferation ability via EGFR regulation and inhibit the activation process of mitogenic signaling in keratinocytes. Additional *in vivo* or *in vitro* investigations are required to verify the *in silico* prediction findings from this study.

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Conflict of Interest

Authors declare there is no conflict of interest in this study.

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