ISSN: 1410-8917
Jurnal Kimia Sains & Aplikasi
e-ISSN: 2597-9914

Jurnal Kimia Sains dan Aplikasi 23 (4) (2020): 117–123

Jurnal Kimia Sains dan Aplikasi
Journal of Scientific and Applied Chemistry

Journal homepage: http://ejournal.undip.ac.id/index.php/ksa

In *Silico* Screening Anticancer of Six Triterpenoids toward miR-494 and TNF-α Targets

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https://doi.org/10.14710/jksa.23.4.117-123

Article Info

Article history:

Received: 8th December 2019
Revised: 2nd April 2020
Accepted: 7th April 2020
Online: 30th April 2020

Keywords:
Akt; cycloartenol; *Ganoderma lucidum*; hepatocellular carcinoma; triterpenoids

Abstract

Hepatocellular carcinoma (HCC) accounts for up to 90% of all primary liver cancers worldwide. Cinobufagin is recognized to inhibit miR-494 as the HCC target. Increased expression of TNF-α results in an inadequate response to liver anticancer drugs. The models in this study were cinobufagin, cycloartenol, and ethyl acetate fractions of *Ganoderma lucidum*, **2–5**. Seven docking targets in this study were Akt, ERK1, ERK2, PI3K, TNF-α, TNFR1, and TNFR2. Cycloartenol and compound **4** comply with Veber's rules, Lipinski's rule of 5, and demonstrate moderate toxicity. The action implies a potential docking target since it produces bond affinities with the compound **2–5** that agree with the IC_{50} in the literature, which is based on in *vitro* experiments. Akt as a receptor target is AZD5363. Cycloartenol shows a low ability to inhibit Akt. Conversely, compound **4** inhibits the Akt better than that of cycloartenol, although it is not as good as cinobufagin and AZD5363. Therefore, compound **4**, a triterpenoid with a basic framework of lanostane has the potential to be an anticancer candidate for the liver.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in the digestive system [1], the fifth most common cancer, causing more than 600,000 mortalities worldwide annually, and the incidence is still increasing [2]. Surgical treatments, such as liver resection and transplantation, are the first-line therapeutic strategies for HCC. However, the postoperative survival rate is only 30–40% at five years, because HCC is a relatively chemo-resistant tumor and highly refractory to cytotoxic chemotherapy [3, 4]. MicroRNA-494 (miR-494) acts as an oncomiR and is involved in tumor development, progression and metastasis, and confers resistance to chemotherapeutic drugs by targeting several molecules in several human cancers. Given the close relationship between miRNAs and the biological progression of various types of cancer, miRNAs are considered as potential novel targets for the therapy of various types of cancer [5]. Tumor necrosis factor (TNF)-α is one of the essential inflammatory cytokines, and it was first identified as an anti-tumor cytokine that induced tumor necrosis. Recent evidence indicated that TNF-α is a central mediator of inflammation, and thus provides a molecular link between chronic inflammation and the development of malignancies [6]. Tan *et al.* [7] reported that TNF-α is positively related to the proliferation and invasion ability of the HCC cells.

Cinobufagin (**1**) (**Figure 1**) is bufadienolide type cardiotonic steroids, a primary active component of Chan Su, which is a traditional Chinese medicine derived from skin and parotid venom glands of toad Bufo *gargarizans Cantor* [8]. Recently, cinobufagin has been demonstrated to induce apoptosis in human leukemia, HCC, and prostate cancer cells. Qi *et al.* [9] reported that cinobufagin induced marked changes in apoptotic morphology and significantly increased the proportion of apoptotic cells of cell line HepG2 via both Fas- and mitochondria-mediated pathways, and a Fas-mediated caspase-10-dependent pathway. The IC_{50} of cinobufagin is 0.17–1.03 μmol/L. Triterpenoids are also believed to form the pharmacodynamic material basis of the demonstrated anti-cancer effects. Chen *et al.* [10] reported that lanostane-type triterpenoids which are typical constituents of *Ganoderma lucidum* compounds **2-4** showed highly cytotoxic toward hepatocellular cancer

cells HepG2 with an IC_{50} of 43.7±1.4, 39.3±1.3, and 41.5±3.2 µM, respectively. Meanwhile, compound **5** exhibits moderate cytotoxicity with an IC_{50} 82.6 ± 5.8 µM. Compound **6** from the ethanol extract of avocado seed showed IC_{50} 12 mg/mL and is safe toward healthy cells [11].

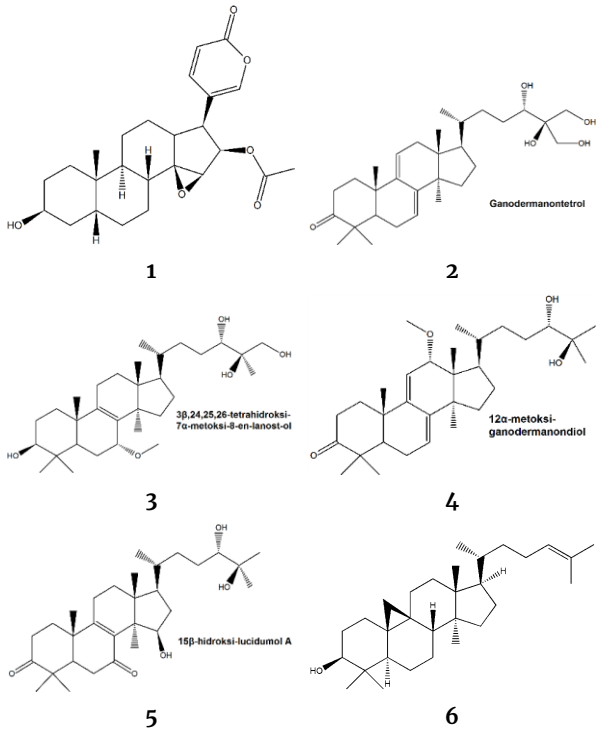


Figure 1. Structures and names of compounds **1–6**

In-silico virtual screening and docking become a significant part of the rapid design and discovery of novel drugs. This study attempted to design and to discover novel drugs for HCC treatment using molecular docking. Molecular docking is a computational procedure for predicting noncovalent binding between macromolecule(s) (as a receptor) and a small molecule (as a ligand) efficiently, start from their unbound structures, structures obtained from MD simulations, or homology modeling, or begins with an unattached structure, the structure obtained from MD simulations, or homology modeling. Previous experiments showed that compound **6** did not optimally decrease the miR-494 expression by targeting ERK2 due to unstable complex conformation [12]. Therefore, the approach of our study was to select an appropriate target of the compounds **2-5** by observing the appropriateness of IC_{50} and to compute the binding affinity to seven receptors, i.e., Akt, ERK1, ERK2, PI3K, TNF-α, TNFR1, and TNFR2. The AZD5363 was to inhibit Akt, SCH772984 to ERK1 and ERK2, GDC-0326 to PI3K, and inhibitor to TNF-α [13, 14, 15, 16]. The purpose of this study is to predict the bound conformation and the binding affinity of compounds **1-6 (Figure 1)** by evaluating the decreasing expression of the miR-494 and the TNF-α. Predicting the bound of small molecules to proteins is of practical importance for it is used to screen virtual libraries of drug-like molecules to lead further drug development. Docking can also be used to predict the bound conformation of known binders when the experimental holo-structures are unavailable [17].

2. Computational Methods

2.1. General

To start with, the ligands (compounds **1-6**) were evaluated for absorption, distribution, metabolism, and excretion (ADME) by Veber's, Lipinski's rules using Drulito references. Toxicity was assessed using TEST software to calculate LD_{50} oral mouse consensus methods to predict pharmacokinetic properties [18]. Before molecular docking studies, the ligand structures were optimized employing GAMESS software using the *ab initio* method (HF/6-31G(d)/C-PCM) [19, 20]. The ligands **1-6** and the selected target proteins PDB files were loaded to AutoDockTools to predict the predominant binding mode with exhaustiveness parameters 32 in triplicate using AutoDock (AD) Vina calculations. The results of docking are the value of ligand affinity that indicate the appropriateness of the ligands inhibit the respective receptors. The outputs were then used to predict the pharmacodynamic properties of the ligands.

2.2. Preparation of Protein Receptor and Molecular Docking

Table 1. The selected target receptor macromolecules used to observe the reducing expression of miR-494 for the treatment of HCC in *Homo sapiens*

	Akt	ERK1	ERK2	PI3K	TNF-α	TNFR1	TNFR2
PDB ID	4GV1	4QTB	4QTA	5DXT	2AZ5	1EXT	1CA9
Mutation	2	-	-	-	-	-	-
Method	XRD	XRD	XRD	XRD	XRD	XRD	XRD
Resolution (Å)	1.49	1.49	1.45	2.25	2.00	1.85	2.30
Inhibitor	AZD 5363	SCH 77984	SCH 77984	GDC-0326	TNF-α Inhibitor	-	-
R work	0.181	0.147	0.158	0.227	0.22	0.203	0.234
Ramachandran outliers	0.000	0.000	0.000	0.000	0.400	0.300	1.100

Protein receptors were downloaded from the RCSB PDB in the form of *.pdb files (**Table 1**). The receptor structure is selected based on species suitability, a minimum number of mutations where XRD is more recommended than that of NMR. Structures that interact with inhibitors were preferred over structures without inhibitors, excellent resolution below 2 Å, *R* work values less than 0.2, and the Ramachandran outliers below 2%. Furthermore, water and the unwanted molecules were removed using a Chimera, then the hydrogen atoms were added to the receptor using AutoDock Tools. The ligand compounds **1–6** structures were drawn through Avogadro molecular editor and visualizer, then optimized via GAMESS software using the *ab initio* method (HF/6-31G(d)/C-PCM) [19, 20].

Ligands in *.pdb format were opened in AutoDockTools, and automatically the gasteiger charges were added. Furthermore, the ligands coordinate rotatable bonds were selected and saved in the *.pdbqt format. Afterward, the receptor coordinates were selected by opening the receptor file and saved in the *.pdbqt format. The grid box was determined according to the coordinates of each original inhibitor of the docking target. The obtained docked complexes were run through ADT software to produce the refinement and rescoring of the rigid body protein-ligand solutions. The best refined

docked protein-ligand complexes from ADT were analyzed, and the complete illustration was generated using Discovery Studio. Finally, the affinity parameters of bonds, hydrogen bonds, and the pharmacophore of the complex formed between ligands and proteins were analyzed [19].

3. Results and Discussion

The result of pharmacokinetic properties to compounds **1 to 6** were evaluated according to Lipinski's rule of 5, Veber's rules, and the toxicity parameter was further assessed through the *in-silico* approach (**Table 3** and **Table 4**, respectively). Table 3 summarizes that all compounds complied with Veber's rule and Lipinski's rule of 5, however, three of six (compounds **2-4**) show that the ClogP of Lipinski's rules is slightly higher than that of compound **5**, and compound **6** shows the highest value (11.921) amongst the ligands. On the other hand, according to toxicity criteria in Table 4 [21], compounds **1** and **2** are more toxic than that of other compounds, while compounds **3-6** are categorized as moderate. Based on the ClogP of Lipinski's rules and toxicity values, compounds **1** and **2** have no potential to be developed as drug candidates. Compounds **3** and **6** also show low absorption and permeability, thus have no potential as drug candidates as well. Therefore, only compounds **4** and **5** demonstrate the potential to be a low-dose drug candidate.

Table 3. Absorption, distribution, metabolism, excretion, and toxicity of six ligands understudy

Ligand	MM (Da)	CLogP	HBA	HBD	NRB	TPSA (Å)	LD_{50} oral rat (ppm)
1	442.24	3.823	6	1	3	85.36	6.17
2	488.35	5.206[a]	5	4	7	97.99	4.08
3	506.40[a]	5.429[a]	5	4	7	90.15	77.06
4	486.37	5.748[a]	4	2	6	66.76	251.98
5	474.37	4.747	4	3	5	77.76	413.67
6	426.39	11.921[a]	1	1	4	20.23	404.87

[a] Out of Lipinski's rule of 5

Table 4. Toxicity classification of LD_{50} oral rat [21]

Toxicity Level	LD_{50} oral rat	Classification
1	≤ 1 mg/kg	Very toxic
2	1–50 mg/kg	Toxic
3	50–500 mg/kg	Moderate toxic
4	500–5000 mg/kg	Mild toxic
5	5-15 g/kg	Practically non-toxic
6	≥ 15 g/kg	Relatively harmless

Evaluation of pharmacodynamic properties was performed by docking with exhaustiveness parameters worth 32 in triplicate using AutoDock Vina calculations. Complex formation is thermodynamically preferable if the binding affinity is low. The binding affinity is directly proportional to the IC_{50} protein-cytokinin. The results of the docking compounds **1-6** to the receptors are summarized in **Table 5**. The docking simulation of compounds **1-6** to ERK1, ERK2, PI3K, TNF-α, TNFR1, and TNFR2 receptors shows relatively high bond affinity as compared to Akt; consequently, it will not be described further. The binding affinity of compounds **2-5**, which roughly shows a tendency similar to the IC_{50} values described by Chen *et al.* [10], is the Akt protein. Hence, the docking discussion will be focused on the Akt protein. The compounds **2** and **3** exhibit similar binding affinity; this case because the AutoDock Vina calculation was only based on molecular mechanics calculations. To improve the calculation, it is necessary to calculate DFT or *ab initio* or other software, such as NAMD using QM/MM calculations.

Akt is a specific protein kinase serine/threonine, which plays an important role in various cellular processes such as glucose metabolism, apoptosis, cell proliferation, transcription, and cell migration. These are important metabolic effects, including uptake of glucose in muscle and fat cells or suppresses neuronal cell death. These effects are often associated with tumor cell survival, proliferation, and invasion [22]. Many attempts have been made to identify Akt inhibitors. The development of competition compounds illustrates the most common approach with ATP or by preventing the formation of active enzymes [23], although Akt inhibiting is still unclear, whether it is ATP-competitive or non-competitive inhibition [24].

The AZD5363 inhibitor exhibits better docking interactions with Akt with the lowest binding affinity (-12.9 kcal mol^{-1}). Interactions are favored by the formation of H- bond with Ala230 in the pyrolopirimidine ring, the base amino group, also interacting with sulfur Met281, and forming hydrogen bonds with the Glu234 side chain and the Glu278 backbone carbonyl [14]. Furthermore, hydrophobic interactions with the central piperidine ring adopt axial conformation.

Table 5. Binding affinity with docking simulations and their suitability with IC_{50}

Ligand	Binding affinity (kcal mol^{-1})							IC_{50}[b] (μM)
	Akt	ERK1	ERK2	PI3K	TNF-α	TNFR1	TNFR2	
Inhibitor[a]	-12.9	-14.7	-14.6	-9.7	-8.8	-	-	-
1	-10.3	-8.8	-8.2	-6.5	-8.8	-6.8	-7.3	1.03
2	-9.2	-9.0	-9.9	-6.9	-7.9	-6.7	-6.8	42.0
3	-9.2	-8.8	-10.0	-7.0	-7.8	-6.6	-6.4	36.5
4	-9.4	-8.1	-8.0	-6.8	-7.9	-6.1	-6.4	4.9
5	-9.3	-8.8	-10.3	-10.3	-8.6	-6.1	-7.4	21.7
6	-8.6	-9.9	-10.2	-7.6	-8.5	-6.7	-6.7	23.8

[a] AZD5363 (Akt), SCH772984 (ERK1 and ERK2), GDC-0326(PI3K), Inhibitor of TNF-α (TNF-α)
[b] Chen *et al.* [10]

The AZD5363 inhibitor exhibits better docking interactions with Akt with the lowest binding affinity (-12.9 kcal mol^{-1}). Interactions are favored by the formation of H- bond with Ala230 in the pyrolopirimidine ring, the base amino group, also interacting with sulfur Met281, and forming hydrogen bonds with the Glu234 side chain and the Glu278 backbone carbonyl [14]. Furthermore, hydrophobic interactions with the central piperidine ring adopt axial conformation.

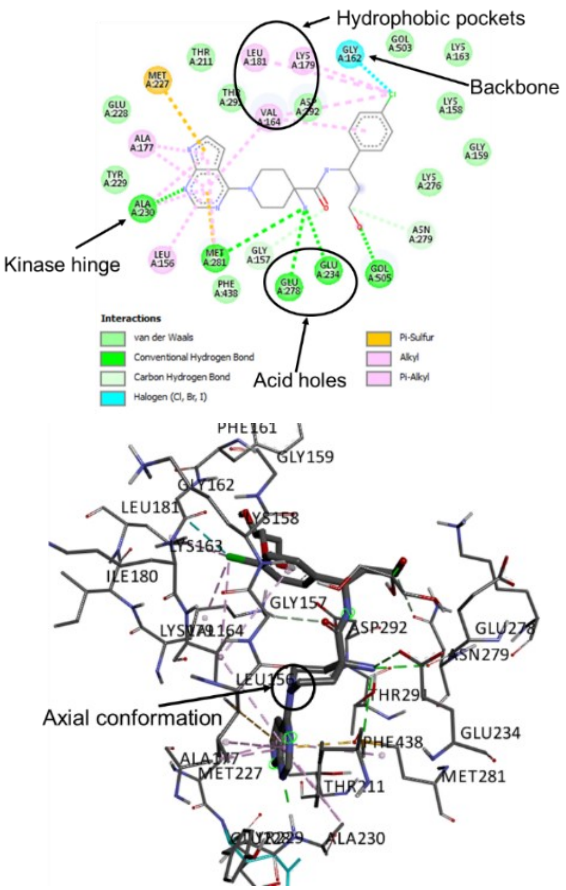
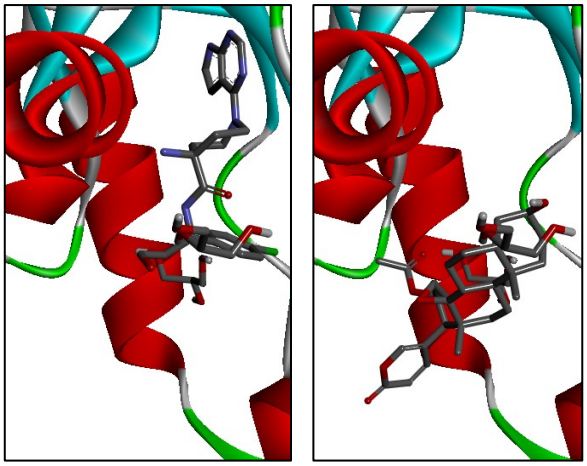


Figure 2. Interaction of Akt and AZD5363 inhibitor

In contrast, the binding affinity of compound **6** is the highest amongst the compounds. The compound **1** inhibits Akt with a binding affinity of around -10.3 kcal mol^{-1}, and the compounds **2-5** tend to have a similar affinity around -9 kcal mol^{-1}. The conformation of AZD5363 inhibition to Akt is shown in **Figure 2**. The pyrolopirimidine ring forms a hydrogen bond to the kinase hinge through the Ala230 residues, and the central piperidine ring adopts axial conformation. The tendency of the axial position is affected by *ortho*-sp^2 nitrogen in the pyrolopirimidine nucleus. This conformation explains a base amino group in an acid cavity formed by Glu234 and Glu278, the *p*-chlorophenyl group in the hydrophobic pocket with the side chains of Lys179, Leu181, Val164, and the backbone of Gly162. Even though the axial position tends to be less energetic than that of the equatorial, however, this conformation optimally interacts between the AZD5363 and the Akt. Besides, the basic amino group also interacts with sulfur of the Met281 and forms hydrogen bonds with the Glu234 side chain and the Glu278 of backbone carbonyl [14]. This case results in a low binding affinity between the AZD5363 inhibitor and the Akt.

Compound **1** forms hydrogen bonds with Gly294 and Gol503, however, it is not as good as AZD5363. Therefore, the binding affinity of compound **1** is slightly higher than that of AZD5363. Besides that, compound **1** only forms π-σ interaction between the dienol and the alkyl groups of this compound to Leu295 and Phe161, respectively, and the van der Waals bond to Val164. On the other hand, this particular compound does not show the acid cavities formed by Glu234 and Glu278 due to the impossible position (**Figure 3**). The conformation of compound **1** to Akt is shown in **Figure 4a**. On the other hand, compound **6** forms π-σ interaction between the alkyls group to Phe161, His194, Leu295, Lys179, and Leu181, as well as van der Waals bonding to Val164 and Gly162 (**Figure 4b**). Additionally, this compound does not show hydrogen bonds, the formation of acid cavities by Glu234, and Glu278. Therefore, no hinges are formed, and the binding affinity of compound **6** is high.

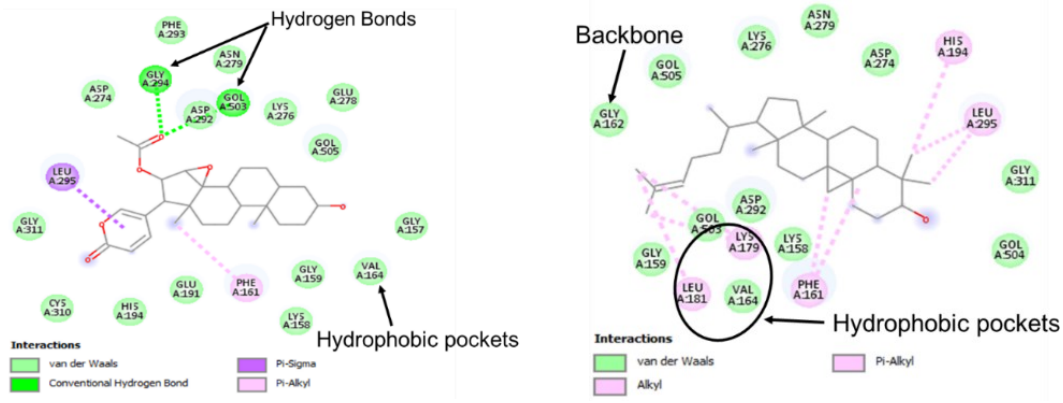


a b

Figure 3. Two different positions of an acid cavity formed by Glu234 and Glu278 between AZD5363 inhibitor (a) and compound **1** (b)

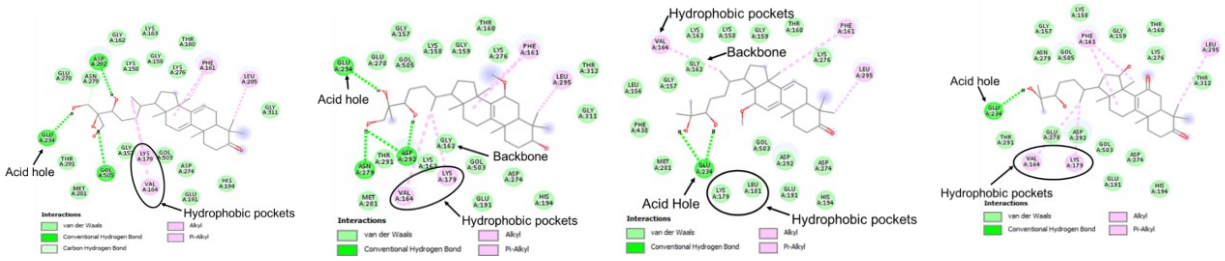
Based on **Figure 5**, Akt interaction with each of compounds **2–5** is alike. The four hydrogen-bonded of these compounds with Glu234 are amino acids that form acid cavities in the hydroxyl group. As for compound **4**, there are two hydrogen bonds with Glu234 in the diol group. Compound **3** forms two other hydrogen bonds with Asn279 and Asp292 with each hydroxyl group. Similarly, compound **2** also forms two other hydrogen bonds with Asp292 and Gol505. Hydrophobic interactions happen between **2**, **3**, and **5** with alkyl on Val164 and Lys179, while compound **4** only interacts with alkyl in Val164, giving a hydrophobic pocket on the side chain. These four compounds also interact with on Leu295 and interaction of π-alkyl with phenyl groups in Phe161. Only compounds **2** and **3** perform van der Waals interaction with Gly162, which are the backbone atoms. Conversely, the compounds **2-5** do not interact with Ala230, which indicates that the position is different from AZD5363 (**Figure 6**). Similar interactions between the four compounds also indicate that their positions are relatively similar.

Akt binding affinity of compound **4** is -9.4 kcal mol^{-1} and is the lower than that of compounds **2, 3,** and **5** in the presence of 2 hydrogen bonds with acid cavities in the diol groups. Even though compound **5** also has a diol group, there is only one hydrogen bond in the acid cavity formed between the hydroxyl and the ketone groups (**Figure 7**), resulting in a binding affinity of -9.3 kcal mol^{-1}. Meanwhile, compounds **2** and **3** are hydrogen-bonded with acid cavities and with other amino acids, which result in a binding affinity to Akt of -9.2 kcal mol^{-1}.



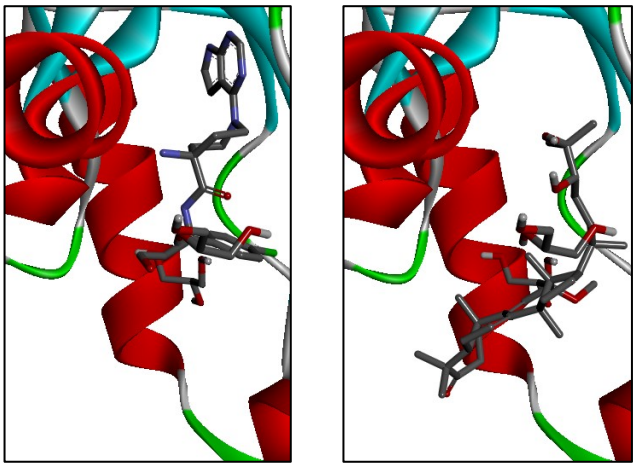
a b

Figure 4. Interaction of Akt with compounds **1** (a) and compound **6** (b)



(a) (b) (c) (d)

Figure 5. Interaction of AKT with compounds of **2** (a), **3** (b), **4** (c) and **5** (d)



(a) (b)

Figure 6. The different interaction to Ala230 between AZD5363 (a) and compound **4** (b)

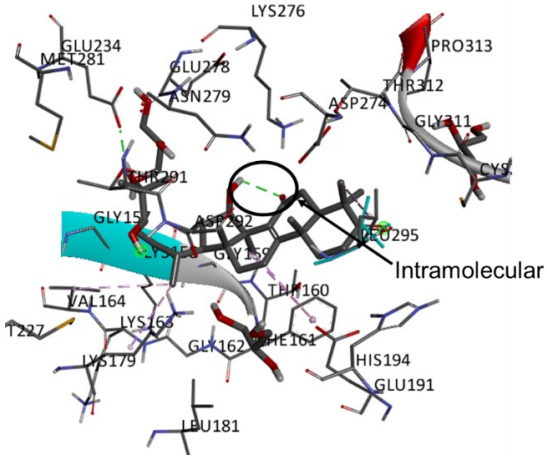


Figure 7. The hydrogen bond between Akt with compound **5**

4. Conclusion

The pharmacokinetic properties of compounds **6** and **4** are in the moderate toxic classification, meet the Veber rules, and Lipinski's rule of 5 but with a ClogP value of more than 5, especially in compound **2** with ClogP of approximately 12. Akt is a potential docking target since it produces binding affinity with compounds **2–5**, which is in agreement with the reported IC_{50} elsewhere. Akt can be inhibited by the interaction of kinase hinges, acid cavities, hydrophobic pockets, and backbone atoms. The results of the *in-silico* study are in line with *in-vitro* research. Base on the pharmacodynamic properties, inhibition of compound **4** toward Akt is better than that of compound **6**, even though the inhibition of compound **4** is not as good as compound **1** and AZD5363. Triterpenoids

with a basic framework of lanostane have the potential to become anticancer liver candidates.

Acknowledgments

The authors acknowledge grant from the Department of Chemistry FMIPA IPB University for funding this experiment.

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