EFFECTS OF DAIDZIN AND ANALOGUE OF *GANODERMA SINENSE* ON BACTERIALLY-EXPRESSED HUMAN HEXOKINASE ISOFORM 2 FOR ANTI-DENGUE DRUG DESIGN

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ABSTRACT: Dengue disease, which is caused by dengue virus (DENV) has been a major worldwide concern, with increased number of cases each year. Currently, there are no specific medications to treat the disease. Hence, there is a dire need to develop novel drugs for disease treatment. Glycolysis is a metabolic pathway that serves as the main source of energy for DENV replication and targeting the pathway is one of the ideal approach to discover new anti-DENV drugs. This paper focuses on the inhibition of human hexokinase isoform 2 (HK2) enzyme, which is one of the important enzymes in glycolysis, in the quest to disrupt DENV replication. In order to search for potential inhibitors, two methods were conducted, which are ligand-based screening and structure-based screening approaches. Docking of Daidzin , which was derived from Kudzu, a Japanese plant, into the active site of HK2 has shown the nearest binding affinity score (-7.94 kcal/mol) to glucose's (GLC), which is -8.15 kcal/mol. Meanwhile, Ethyl (2*R*)-2-[[3-[2-[(4-methylbenzoyl) amino]ethyl]-[1,2,4]triazolo[4,3-b]pyridazin-6-yl]sulfanyl]butanoate (Ethyl 2(R)), a compound which is the analogue of ((22E, 24R)-6β-methoxyergosta-7, 9(11), 22-triene-3β,5α-diol) or compound 2 from *Ganoderma sinense* obtained from ligand-based screening was also docked into the binding site of HK2, showing a binding affinity score of -8.43 kcal/mol. Both docking was conducted by using AutoDock4 software at virtual screening phase. These compounds were further analysed in an inhibition assay to determine the effects of these potential naturally-derived inhibitors on the activity of HK2. The outcome from the inhibition studies showed that both compounds exhibited substantial inhibition on the activity of HK2 enzyme, where Daidzin, at 0.5 mM, resulted in HK2 remaining activity of 87.28%, while Ethyl (2R) resulted in 70.09% of HK2 remaining activity at 0.5 mM concentration. The results also indicate that as the concentration of these compounds increased, the percentage of remaining enzyme activity decreased. In conclusion, this study has served as a platform for the development of anti-dengue drugs based on naturally-derived compounds, which is anticipated to be a safer option for dengue treatment.

KEY WORDS: *Dengue virus, glycolysis, human hexokinase isoform 2, in silico drug design, enzyme inhibition.*

1. INTRODUCTION

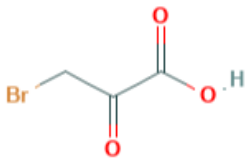
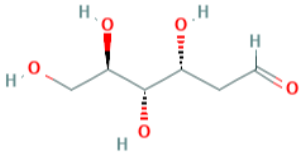
Dengue virus (DENV) is a mosquito-borne disease originated from the genus Flavivirus of the family Flaviviridae, which consists of four serotypes ranging from DENV 1, DENV 2, DENV 3 and DENV 4 [1]. This virus is transmitted *via Aedes aegypti*

mosquito, and once a person has recovered from the first infection by one of the DENV virus serotype, the person may have developed immunity towards the specific serotype, but not long-term protections from other serotypes. Infections by DENV may cause mild fever, however if left untreated, severe situations such as dengue shock syndrome may occur, which possibly lead to death [2]. Until now, Dengvaxia is the only vaccine available in the market to tackle this problem. However, due to the complexity of the virus, this vaccine is not reliable enough and had caused dispute in the Philippines, when the children who received the vaccination died due to complications caused by the vaccine [3]. Hence, efforts to cure dengue remains imperative and should be a priority in research.

Virus is a particle that requires a host in order to replicate, since it does not possess its own metabolism. Once DENV enters a host, it evolves and induces the host's glycolytic pathway, which subsequently increase glucose uptake and lactic acid production in the host's system, mainly due to the viral replication process [4,5]. Glycolytic pathway is a metabolic pathway that converts glucose into pyruvate in ten steps, with the human hexokinase 2 (HK2) as its first rate-limiting enzyme, governing the phosphorylation of glucose into glucose-6-phosphate [6,7]. Thus, HK2 enzyme has been suggested as a target for drug design and development. Currently, there are several known HK2 inhibitors that had been identified; Metformin (Met), 2-Deoxyglucose (2-DG) and 3-Bromopyruvate (3-BP), in which only 2-DG and 3-BP (Table 1) were proven to successfully block the glycolysis flux [8]. However, these inhibitors have downsides as they exhibited adverse side effect to patients, for example, during intravenous infusion, 3-BP causes burning venous sensation and rapid inactivation by thiol groups of glutathione and proteins [9]. Furthermore, 3-BP also tend to be less selective to tumor tissues. Meanwhile, in the case of 2-DG, clinical studies have reported that several serious side effects were reported to be developed by patients such as reversible hyperglycemia, gastrointestinal bleeding, and reversible grade 3QTc prolongation after drug consumption at a dosage of more than 63mg/kg [10].

In this paper, potential HK2 inhibitors based on naturally-derived compounds had been screened *via* ligand-based and structure-based approaches. For the ligand-based method, ((22E, 24R)-6β-methoxyergosta-7, 9(11), 22-triene-3β,5α-diol) or compound 2, which was a compound extracted from *Ganoderma sinense* was used as a reference molecule [11]. Meanwhile, a natural compound database was used to screen for potential inhibitors for the subsequent structure-based method. The purpose of virtual screening is to search for molecules that can potentially bind with the target enzyme, creating an enzyme-ligand complexes that will give the most favourable binding energy [12,13]. The potential inhibitors then were chosen based on its binding energy with the target protein. Subsequently, the ProTox-II webserver was used to predict the toxicity of the selected compounds prior to the inhibition study, which was conducted by using a coupled-enzymatic assay to determine the activity of HK2 in the presence of the potential inhibitors. This is based on the absorbance of NADPH at 340 nm wavelength measured using UV-Vis spectrophotometer, which is to measure the concentration of NADPH in the aqueous solution, where the higher the phosphorylation of glucose, the higher the absorbance peak and vice versa [14]. The increased level of NADPH indicates the rise of glucose-6-phosphate, which will eventually inhibit hexokinase enzyme, as this enzyme is inhibited by its own product, glucose-6-phosphate [15].

Table 1: Known HK2 inhibitors that have been proven to block the glycolytic flux.

Name	Structure	Chemical formula	References
3-Bromopyruvate (3-BP)		$C_3H_3BrO_3$	[8]
2-Deoxyglucose (2-DG)		$C_6H_{12}O_5$	[8]

2. MATERIALS AND METHODS

2.1. Virtual Screening Analyses:

USR-VS webserver was utilized in order to conduct the ligand-based method by using compound 2 ((22E, 24R)-6β-methoxyergosta-7, 9(11), 22-triene-3β,5α-diol) from *G.sinense* as a query molecule. This programme was able to screen 93.9 million 3D conformers of 23 million molecules in about 2 seconds [16]. The similarity score was ranked based on the compounds that have similar configurations with compound 2 from *G.sinense*, and top ten compounds out of 100 from the hits file were subsequently being docked in the structure-based programme. For the structure-based method, several natural compound databases were utilized to search for prospective inhibitors, where 20 potential anticancer molecules that follow the Lipinski's Rule of Five were chosen to be docked into the HK2 structure. Next, docking analyses between the ligands and HK2 were performed by using AutoDock software version 4.2, to obtain the binding energy. Finally, toxicity test was conducted for all compounds by using ProTox-II webserver.

2.2. Inhibition Analyses with Selected Compounds:

An inhibition study by using a coupled-enzymatic assay was being conducted to analyse effects of the virtually-screened compounds on HK2 activity by measuring the increased absorbance of NADPH.

Determination of HK2 activity was carried out in a 1 mL volume containing 47 mM Trizma base buffer (pH 7.4), 42 mM $MgCl_2$, 0.8 mM NADP, 5.0 mM ATP, 4.17 mM glucose, 2500 U/ml G6PDH, and dH_2O. These solutions were incubated at room temperature for one hour to equilibrate the assay before HK2 was added to the solution. The samples absorbance was then measured every 1 minute, for 15 minutes. Consequently, the same experimental setup for HK2 inhibition studies were prepared, yet in the presence of compounds at different concentrations, which are 0.5 mM, 1.0 mM and 2.0 mM, respectively. The positive control in this experiment is 3-BP, as it is a known inhibitor of HK2, while the negative control is the reaction mixture in the absence of inhibitors. All

readings were taken by using UV1200 single beam UV-Vis Spectrophotometer (Perkin Elmer SP-UV 500 Series) in duplicate and the average value of absorbance was recorded for the inhibition analyses.

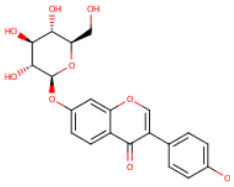
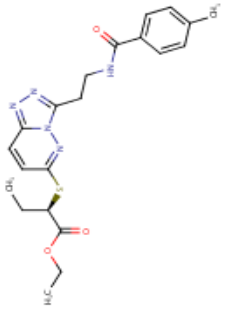
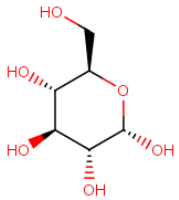
3. RESULTS AND DISCUSSION

3.1. Virtual Screening Analyses

Two potential inhibitors that adhered to the Lipinski's Rule of Five (Table 2) were selected, following the virtual screening analyses. Lipinski's Rule of Five served as a rule of thumb for oral drug design, where in order for a compound to be designed as an orally-active drug, it should not have more than five hydrogen bond donors, have less than ten hydrogen bond acceptors, molecular mass less than 500 Da and xlogP value not exceeding five [17]. The binding energy for these compounds are closer to the binding energy of glucose substrate with HK2, indicating that it will fit well when bound to HK2, since glucose will solidly bind to the protein as it serves as HK2 substrate. Hence, these two compounds were expected to bind well with HK2. Toxicity prediction test that showed lethal dose value and the class for both compounds, which are Daidzin and Ethyl (2R) was done by ProTox-II [18]. The toxicity classes are as stated below:

- Class I: fatal if swallowed ($LD50 \leq 5$)
- Class II: fatal if swallowed ($5 < LD50 \leq 50$)
- Class III: toxic if swallowed ($50 < LD50 \leq 300$)
- Class IV: harmful if swallowed ($300 < LD50 \leq 2000$)
- Class V: may be harmful if swallowed ($2000 < LD50 \leq 5000$)
- Class VI: non-toxic ($LD50 > 5000$)

Table 2: Potential inhibitors derived from the computer-aided virtual screening analyses

Compound Name	Compound Structure	Binding Energy (kcal/mol)	ProTox-II Prediction (LD50, mg/kg)	H-bond donor (<5)	H-bond acceptor (<10)	MW (<500 da)	XlogP (<5)
Daidzin		-7.94	3100	5	9	416.4	0.7
Ethyl (2*R*)-2-[[3-[2-[(4-methylbenzoyl)amino]ethyl]-[1,2,4]triazolo[4,3-b]pyridazin-6-yl]sulfanyl]butanoate		-8.43	2000	1	7	427.5	3.4
Glucose *Marker		-8.15	12054.41	5	6	180.2	-2.6

3.2. Inhibition Analyses with Selected Compounds:

The activity of HK2 in the presence of Daidzin and Ethyl (2R) was measured to evaluate the inhibition effects of the virtually-screened compounds on HK2 activity. The positive control used in this study is 3-BP, while the negative control is the activity of enzyme in the absence of any inhibitor compound. Samples were tested at three different compound concentrations, which are 0.5 mM, 1 mM and 2 mM. The absorbance value of each sample was recorded to determine the specific activity of HK2. Fig. 1 shows the effects of these compounds on the activity of HK2 at the three different concentrations.

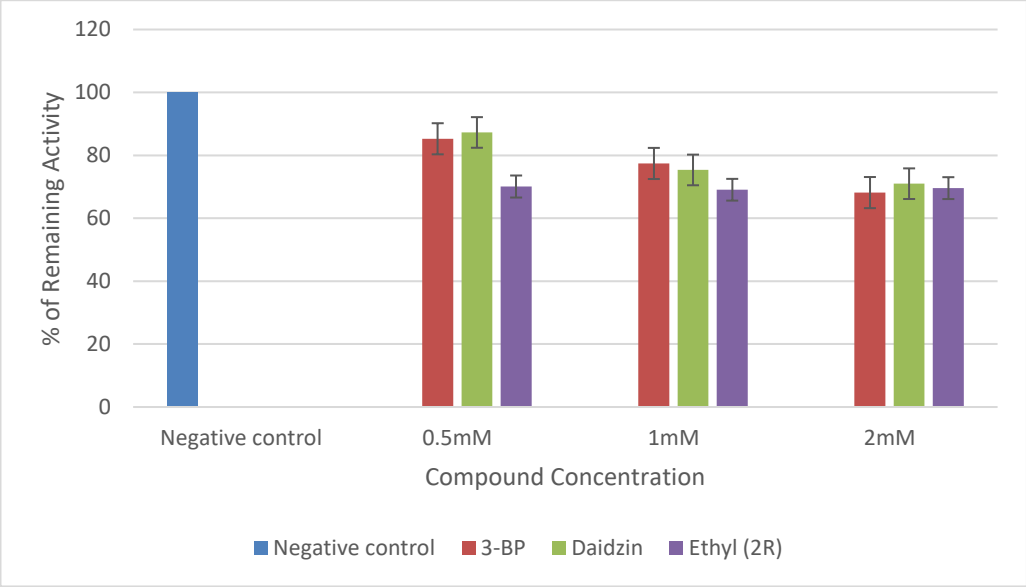


Fig.1 Percent of remaining HK2 activity versus compound concentrations between the positive control (3-BP), Daidzin and Ethyl (2R) at 0.5mM, 1mM and 2mM, consecutively.

The results from Fig. 1 showed that the percentage of enzyme inhibition increased with higher concentrations of compounds. The potential inhibitors, Daidzin and Ethyl (2R) (analogue of compound 2 from *G.sinense)* at 0.5 mM concentration showed promising results, where the percentage of remaining activity of HK2 in the presence of 0.5 mM Daidzin and Ethyl (2R) were determined to be 87.28% and 70.09%, respectively. These values are relatively close to the percentage of remaining activity of the positive control (3-BP), which is 85.27% at the same concentration. Meanwhile, at a higher concentration (2 mM), the percentage of remaining HK2 activity was substantially lower, which is only 70.99% for Daidzin and 69.58% for Ethyl (2R). It is noteworthy to note that the binding energy of Daidzin is -7.94 kcal/mol, while the binding energy of Ethyl (2R) is -8.43 kcal/mol. These compounds were chosen based on the binding energy that gave the nearest value to glucose, which is -8.15 kcal/mol. It is clear that the trend of inhibition between the control (3-BP) and Daidzin is decreasing linearly with increased concentrations. At 2 mM concentrations, Daidzin and 3-BP have almost similar remaining activity, in which both had the lowest remaining activity of 70.99% and 68.16% respectively, as shown in Fig. 1, which may be due to the common structures that both compounds possess that have been measured using MCS Tanimoto with the value of 0.1212 and MCS size of 4. However, the value of similarity is not that great, explaining the reason why the inhibition shown by 3-BP and Daidzin are distinctive. Meanwhile, for Ethyl (2R), the percentage of remaining enzyme activity slightly increased at 2 mM inhibitor concentration.

From previous studies that had been conducted by Wu et. al, (2018), compound 2 that was derived from *G. sinense* had shown high binding affinity to HK2, with kD value equivalent to 114.5± 2.7μM, making it a potential drug candidate for cancer therapy by targeting HK2 [11]. Therefore, in this study, the analogue of compound 2, which is Ethyl (2R), as well as Daidzin have been proven to have potentials for naturally-derived anti-dengue drug development, specifically inhibiting HK2. However, the comparison between 3-BP (control) and Daidzin is more favorable, because the toxicity test results predicted by the ProTox II webserver have shown that Daidzin is less toxic than Ethyl (2R), even though

Ethyl (2R) exhibited better inhibition effects. This is further supported by the consistent results that showed a decreasing trend of remaining activity of Daidzin, compared to Ethyl (2R), where the percentage of remaining enzyme activity increased slightly at 2mM compound concentration.

4. CONCLUSION AND REMARKS:

The results obtained from this study concluded that Daidzin, as well as Ethyl (2R), the analogue of compound 2 ((22E, 24R)-6β-methoxyergosta-7, 9(11), 22-triene-3β,5α-diol) can be regarded as inhibitors of HK2, showing substantial inhibition at 0.5 mM concentration. The results have made it evident that HK2 activity decreased, with higher inhibitor concentrations. Overall, this study has shown promising inhibition results, and further evaluations of these compounds as potent HK2 inhibitors have yet to be conducted to discover novel cure for dengue in the future.

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