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In-Silico Identification and Evaluation of Dual Inhibitors of Glucoamylase and α-Amylase as Potential Treatment for Diabetes

Loh Hui Xuan[a], and Syazwani Itri Binti Amran[a]*
[a]Department of Biosciences, Faculty of Science, Universiti Teknologi Malaysia, 81310 UTM Skudai, Johor, Malaysia
*Corresponding author: syazwaniitri@utm.my

Abstract
Diabetic patients are susceptible to other illnesses leading to serious health complications and increased consumption of medications. To reduce pill burden and decrease the risks of adverse drug-drug interactions due to taking in too many medications, designing a dual inhibitor drug will be the best solution, to achieve the same target result. Glucoamylase and α-amylase enzymes play a role in producing free sugars from carbohydrate digestion contribute to increased glucose level in the blood. Hence, inhibition of these enzymes' activity can act as efficient targets for diabetes and obesity by blocking the production of free sugars into the bloodstream could decrease the blood glucose level. This study focuses on the identification and evaluation of dual inhibitors of glucoamylase and α-amylase as a potential treatment for diabetes, using virtual drug screening method. To identify the potential pharmacophores, 24 compounds were screened using molecular docking method using AutoDock Tools 1.5.6. 2AC12 compound, a novel compound redesigned from AC12 (acarbose 7-phosphate) was found to be the potential dual inhibitor, with the lowest free binding energy of -11.12 kcal/mol in glucoamylase, and -8.99 kcal/mol in α-amylase in comparison to the reference compound; acarbose (-8.64 kcal/mol) and montbretin A (-10.10 kcal.mol) for respective enzymes. Findings from structure-activity relationship analysis shows that 2AC12 can make interactions with the active binding pockets of both target enzymes, and have great potential to be the dual inhibitor for diabetes treatment. From the physicochemical and pharmacokinetic analysis of the 2AC12 compound, it is suggested that this compound is best delivered in prodrug form using oral route of administration for better efficacy of the drug in the treatment of diabetes.
Keywords: Dual inhibitor; glucoamylase; α-amylase; acarbose; potential diabetes treatment.

Introduction

According to WHO, the number of patients diagnosed with diabetes boosted from 108 million in the year of 1980, to 422 million in the year of 2014, and it was shown that there was a 3% increase in diabetes mortality rates by age, between the year of 2000 and 2019 [1]. Generally, diabetes is a heterogenous and progressive disease, which may also lead to other life-threatening health complication and increases the prevalence of getting infections, it is important to look for a new way of drug treatment to handle this problem. For patients with underlying diabetic problems who got infected by a virus or other disease, normal treatment such as insulin injection will not be effective anymore, as their diabetic condition may become worsen because of the infection [2]. Hence, treatment by using enzyme inhibitors may be a potential treatment for this condition, by reducing the production of glucose by blocking carbohydrate metabolism.

There is a great relationship between glucoamylase and α-amylase with diabetes, in which they play a role in producing free glucose from starch. α-Amylase is an extracellular endo-acting enzyme, liberating α-limit dextrin as the product. The catalysis process of α-amylase involves the catalysis of α-1,4 glucan linkages in starch to produce maltose and maltotriose, producing free sugars. Glucoamylase is an inverting exo-acting starch hydrolase, hydrolysing the α-1,4 glycosidic bonds from the non-

reducing ends of starch (dextrin/ maltose/ maltotriose/ glucose oligomers), thus releasing free sugars [3] [4]. Since both enzymes are producing free sugars as by-products, they can act as a conducive target for diabetes and obesity, whereas glucoamylase and α-amylase inhibitors can be used as a carbohydrate blocker, blocking the production of free glucose into the bloodstream [5].

A dual inhibitor is a compound that couples two different desired pharmacological (inhibitory) actions at a similar efficacious dose [6]. The reason why 'dual inhibitor' has become an interest in this study is that both target enzymes are carrying out an almost similar catalytic mechanism, and both are producing free glucose. In order to block the carbohydrate hydrolysis chain, both enzymes must be inhibited. If we design the enzyme inhibitory drug separately, the patients will need to take two types of drugs to achieve a similar carbohydrate-blocking effect. To reduce pill burden and decrease the risks of adverse drug-drug interactions due to taking in too many medications, designing a dual inhibitor drug will be the best solution, to achieve the same target result [7].

Until now, there are still a limited number of approved enzyme inhibitors available in the market. To accelerate the rate of successful treatment of diabetes, the exploration of natural compounds as a source of new enzyme inhibitors could be a potential revenue for diabetic treatment. Since the drug discovery process is a long-drawn-out process, *in-silico* identification and evaluation of drug compound is then extremely important to ensure that the drug is safe and to make sure that the drug designed and produced can bring into play its greatest effectiveness to their consumers. This method is a computational approach to anticipate the drug activity by fitting chemical structures to protein targets, to find out their hit candidates [8]. The details of this method will be described further in the following chapters and sections.

Research Methodology

Design and Preparation of Compounds

Twenty-four ligands (6 alpha-acarbose analogues, 12 acarvosine analogues and 6 montbretin A analogues) were retrieved from the control compounds from glucoamylase (2QMJ) and α-amylase (4W93). The analogues were redesigned using the Avogadro software, from four-ring alpha acarbose to two-ring acarvosine analogues, by drawing and optimizing the energy of the structure, then the structure was exported into PDB format prepared for the following molecular docking steps [9].

Molecular Docking and Binding Profile Analysis

Twenty-four ligands (6 alpha-acarbose analogues, 12 acarvosine analogues and 6 montbretin A analogues) together with 3 controls (acarbose co-crystal ligand, alpha-acarbose from PubChem ID: 445421, and montbretin A co-crystal ligand) were docked into 2QMJ and 4W93, after removing existing water molecules and bonded ligands from the crystal structure, using AutoDock 4.2 [10]. Four ligand compound that achieved a lower or equivalent free binding energies to the control compound were selected for further structural activity relationship analysis, by studying the superimposition and amino acid binding in the active binding site of both 2QMJ and 4W93, using the PyMOL software and BIOVIA discovery studio [11].

Drug-like Properties and Pharmacokinetics Prediction

The compound that has the potential to be a treatment of diabetes with dual inhibitory action to both glucoamylase and α-amylase was further analysed using SwissADME web tool to look for the pharmacokinetic and drug-likeness properties of the compound [12].

Results and Discussion

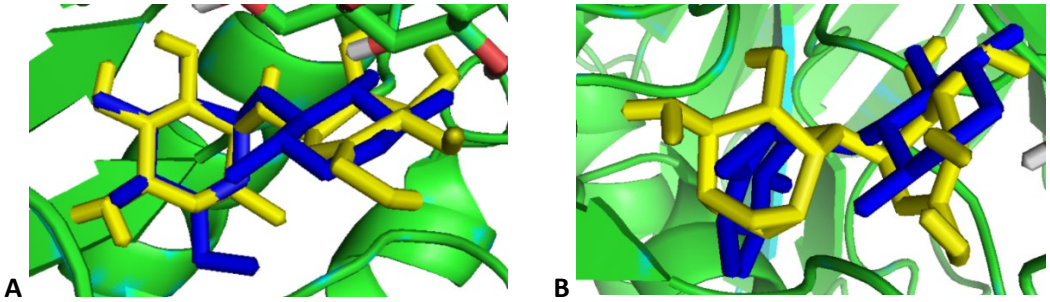
The objective of this study is to identify a potential chemical scaffold that has a high affinity to both glucoamylase (PDB ID: 2QMJ) and α-amylase (PDB ID: 4W93) enzymes. A library compound was set comprising of four-ring alpha-acarbose analogues, two-ring acarvosine analogues and montbretin A analogues. We hypothesized that a potential dual inhibitor will demonstrate high binding affinity to both target enzymes. To study the binding affinity of a potential compound, the parameters involved in the

analysis include the free binding energy, and binding profiles, to find out the similarities in the amino acid residues involved in the binding pockets of both glucoamylase and α-amylase, between the library compounds and the selected control docking models.

The binding interactions between glucoamylase (PDB ID: 2QMJ) with the two-ring acarvosine analogues that demonstrated higher or equivalent binding affinity as the acarbose co-crystal ligand were analysed. Table 1 displayed the free binding energies and amino acid binding interactions between the selected compounds with glucoamylase (PDB ID: 2QMJ), while Figure 1 illustrated the binding mode of the acarbose co-crystal ligand with the compounds in the active binding pocket of glucoamylase. Figure 2 also showed the 2D schematic diagram of the binding network of the selected compound with the amino acids involved in the ligand-protein complex of glucoamylase.

Table 1: Free binding energies and the amino acids binding interactions of the selected ligand compounds in the binding pocket of glucoamylase (PDB ID: 2QMJ)

Ligand Compound	**Acarbose Control**	**2AC1**	**2AC6**	**2AC8**	**2AC12**
Free Binding Energy (kcal/mol)	-8.64	-10.17	-10.04	-8.22	-11.12
Conventional Hydrogen Bond	ASP327 ARG526 ASP542 ASP203 HIS600	ASP327 ARG526 ASP542 ASP203	ASP327 ARG526 ASP203 ASP443 TRP406	GLN 603 ARG526 ASP542 ASP203 TYR299	ASP327 ASP542 ASP443 TRP406
Attractive Charge					ASP327 ASP542 ASP443 PHE575 ASP571
Hydrophobic Interactions	Sulfur-X: MET444	Alkyl: MET444 Pi-Alkyl: TRP406			Pi-Alkyl: PHE575 TYR299



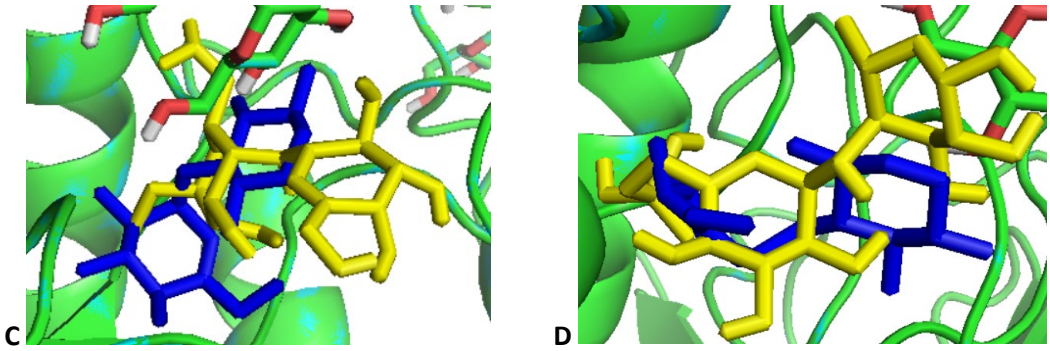


Figure 1 Binding mode of the acarbose co-crystal ligand (Blue) with the compounds in the active binding pocket of glucoamylase (PDB ID: 2QMJ) with the selected compound (Yellow), (A) 2AC1, (B) 2AC6, (C) 2AC8 and (D) 2AC12.

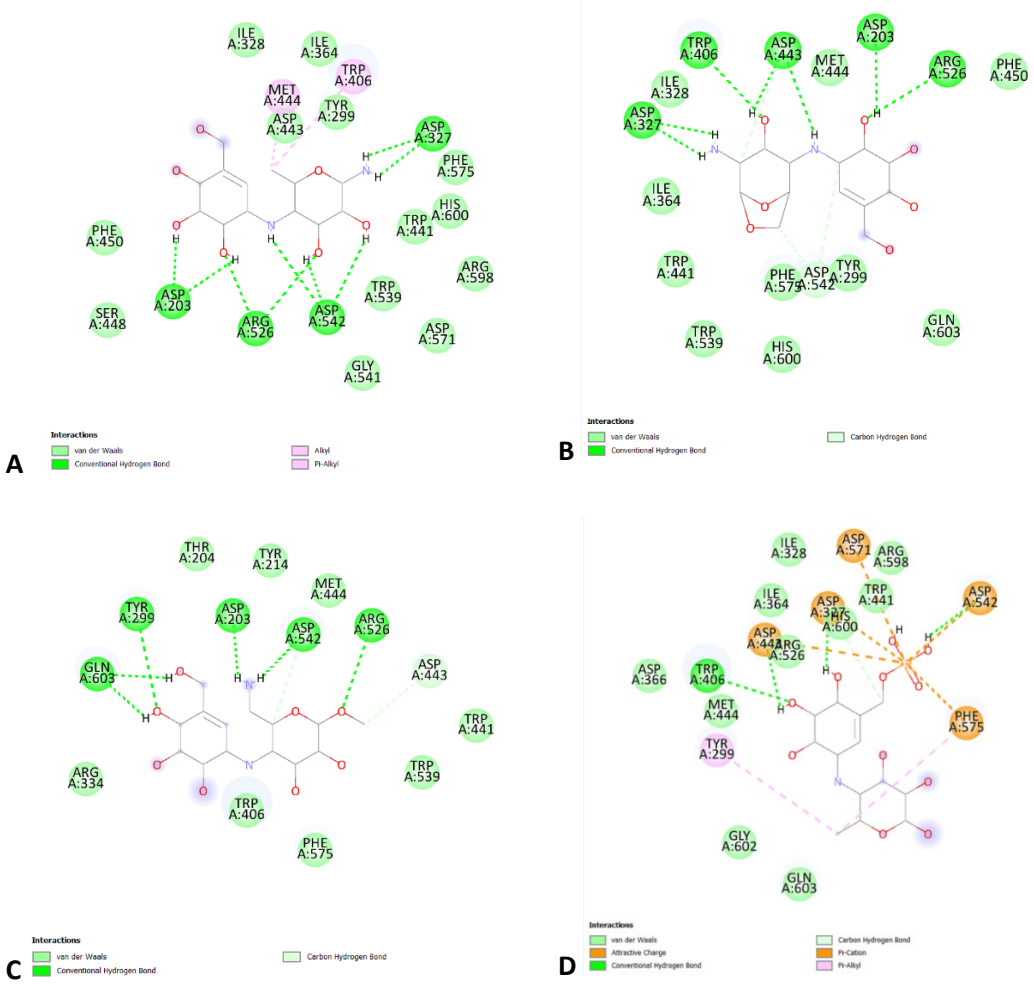


Figure 2 The 2D schematic diagram of the selected compound, (A) 2AC1, (B) 2AC6, (C) 2AC8 and (D) 2AC12, showing the binding network within the glucoamylase (PDB ID: 2QMJ) binding pocket generated using BIOVIA Discovery Studio 4.0 (DS 4.0). The colour codes used in the molecular interactions are represented as follows: hydrogen bond (green-dotted line), van der Waals (medium light green-dotted circle), attractive charges (orange-dotted line), pi-cation bond (orange-dotted line), alkyl bond (pink-dotted line), pi-alkyl bond (pink-dotted line) and water molecules (blue circle).

By comparing the results of library compound (Table 1), there were 4 two-ring acarvosine unit analogues compounds that have free binding energy lower or near to the two-ring control compound, which was 2AC1 (-10.17 kcal/mol), 2AC6 (-10.04 kcal/mol), 2AC8 (-8.22 kcal/mol), and 2AC12 (-11.12 kcal/mol). These compounds showed a higher or similar binding affinity with the binding pocket of glucoamylase (PDB ID: 2QMJ) when compared with the control (acarbose co-crystal ligand), which achieved a free binding energy of -7.01 kcal/mol and an estimated inhibition constant of 0.463μm. Among the 24 library compounds, 2AC12 showed the lowest free binding energy (-11.12 kcal/mol), and the lowest estimated inhibition constant (0.007μm), which indicates that 2AC12 has the highest binding affinity and potency to the glucoamylase binding pocket.

In the comparison of the selected docking models with the original substrate of the target protein, in the catalytic mechanism of glucoamylase (PDB ID: 2QMJ), which hydrolyses the linear α-1,4-linked and branched α-1,6-oligosaccharide substrates as the original substrate, there are six amino acids involved in the active binding pocket. The amino acid residues are HIS600, ASP327, ARG526, ASP203, TRP406, and PHE450 [13]. From Figure 25, we can observe that there were similarities in the amino acid binding in all 2AC1, 2AC6, 2AC8 and 2AC12. Out of the four structures, 2AC12 recorded the most binding interactions with the active binding pocket of glucoamylase, and having 2 amino acids residues overlapped with the original substrate of glucoamylase. The overlapped interactions are the conventional hydrogen bonding with ASP327 and TRP406, and also attractive charges with ASP327. The presence of the overlapping of amino acid residues in both binding interactions indicates that 2AC12 can bind and inhibit the glucoamylase enzyme, hence blocking the digestion of carbohydrates and reducing the release of free sugars into the bloodstream.

On the other hand, the binding interactions between α-amylase protein (PDB ID: 4W93) with the two-ring acarvosine analogues that demonstrated higher or similar binding affinity as the co-crystal ligand (acarbose) were analysed. Table 2 displayed the free binding energies and amino acid binding interactions between the selected compounds with α-amylase (PDB ID: 4W93), while Figure 3 illustrated the binding mode of the acarbose co-crystal ligand with the compounds in the active binding pocket of α-amylase. Figure 4 also showed the 2D schematic diagram of the binding network of the selected compound with the amino acids involved in the ligand-protein complex in α-amylase.

Table 2: Free binding energies and the amino acid binding interactions of the selected ligand compounds in the binding pocket of α-amylase (PDB ID: 4W93)

Ligand Compound	**Acarbose Control**	**2AC1**	**2AC6**	**2AC12**
Free Binding Energy	-7.01	-6.81	-6.16	-8.99
Conventional Hydrogen Bond	GLU233 LYS200 ILE235 GLU240	GLU233 LYS200 ILE235 GLU240	GLU233 ASP300 ASP197	HIS305 ASP300 ILE235
Attractive Charge				ASP300
Hydrophobic Interactions		**Alkyl:** LEU162 **Pi-Alkyl:** HIS201		**Alkyl:** LEU162 **Pi-Alkyl:** HIS201

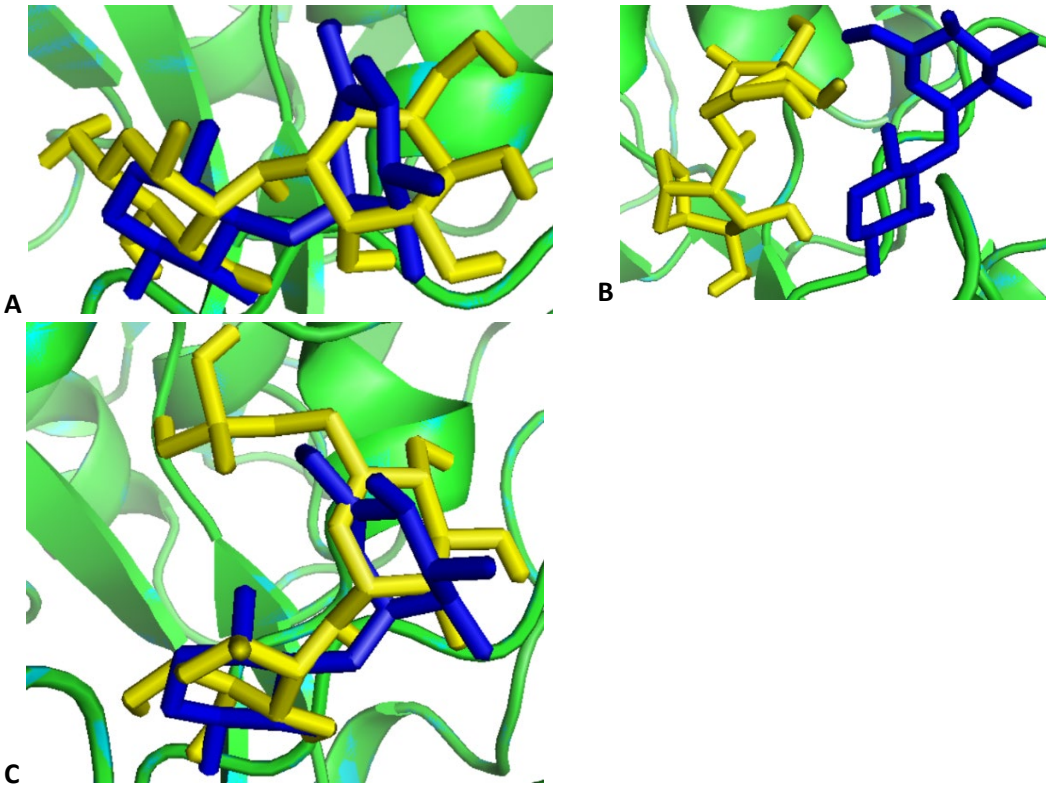
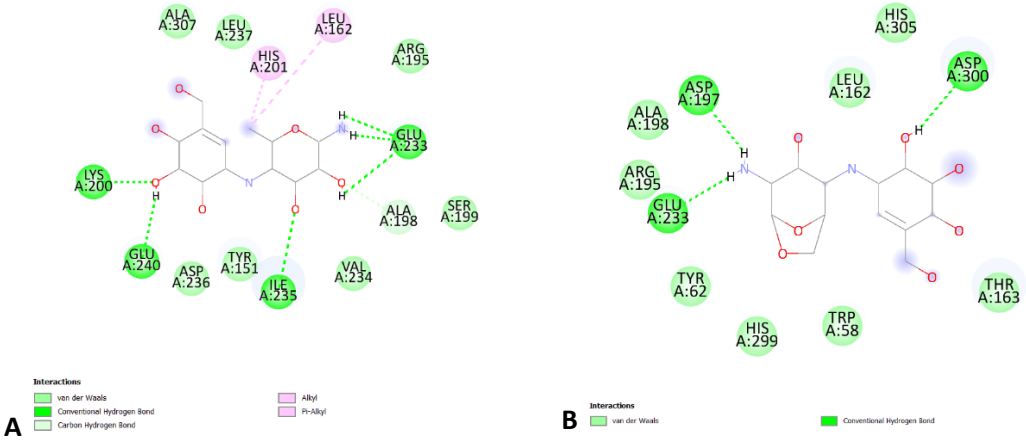


Figure 3 Binding mode of the Acarbose co-crystal ligand (Blue) with the compounds in the active binding pocket of α-amylase (PDB ID: 4W93) with the selected compound (Yellow), (A) 2AC1, (B) 2AC6, and (C) 2AC12.



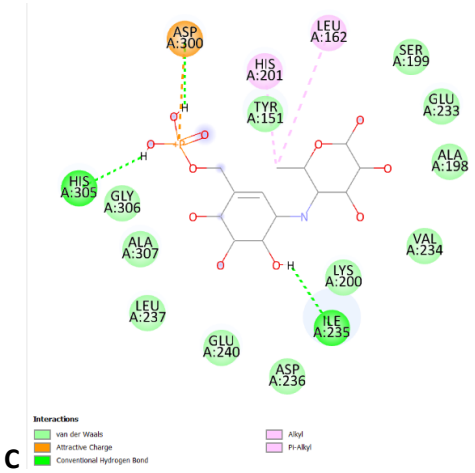


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Figure 4 The 2D schematic diagram of the selected compound, (A) 2AC1, (B) 2AC6, and (C) 2AC12, showing the binding interactions within the α-amylase (PDB ID: 4W93) binding pocket generated using BIOVIA Discovery Studio 4.0 (DS 4.0). The colour codes used in the molecular interactions are represented as follows: hydrogen bond (green-dotted line), van der Waals (medium light green-dotted circle), attractive charges (orange-dotted line), alkyl bond (pink-dotted line) pi-alkyl bond (pink-dotted line) and water molecules (blue circle).

Referring to Table 2 above, the molecular docking of ligand compounds into α-amylase (PDB ID: 4W93), only 3 two-ring acarvosine unit analogues showed a lower or equivalent free binding energy, which was 2AC1 (-6.81 kcal/mol), 2AC6 (-6.16) and 2AC12 (-8.99 kcal/mol), as compared to the free binding energy value for acarbose co-crystal ligand control (-7.01 kcal/mol). The estimated inhibition constant for acarbose co-crystal ligand control was 7.27μm. After comparison with all the library compounds, 2AC12 had the lowest free binding energy (-8.99 kcal/mol) indicating the highest binding affinity to the binding pocket of α-amylase, and the lowest estimated inhibition constant (0.257μm) which contributed to the highest potency with the target protein among all the 27 library compounds.

In contrast to the selected docking models with the original substrate of α-amylase (PDB ID: 4W93), which plays a major role in hydrolysing endo α-1,4- glucan linkage in starch, there are three main catalytic residues involved in the active binding pocket. The amino acid residues are ASP197, GLU233 and ASP300 [14]. By comparing the ligand compound with the lowest free binding energy, which is 2AC12 with a free binding energy of -8.99 kcal/mol, there is one amino acid residue (ASP300) same as the binding of the original substrate to α-amylase. There are two types of interactions formed on ASP300 residue with 2AC12 compound, the conventional hydrogen bonding, and attractive charges, which are also known as the salt bridge interactions. From this observation, the presence of the same amino acid residue involved in the binding interaction of the original substrate and 2AC12 docking model indicates that 2AC12 can bind and inhibit the α-amylase protein. The inhibition of α-amylase will result in the blockage of starch hydrolysis into the production of maltose and maltotriose, thus delaying the release of free sugars into the bloodstream of a diabetic patient.

From the analysis, it can be inferred that the most potential dual inhibitor that can be used as a potential treatment for diabetes is the 2AC12 compound. The structure of this 2AC12 compound is new, and still not available on the current online databases. The structure of 2AC12 is retrieved from AC12, acarbose 7-phosphate (PubChem ID: 101998608). Generally, the structure of acarbose 7-phosphate is greatly similar to the structure of alpha-acarbose, just differs in the addition of phosphate ions on the acarvosine unit of alpha-acarbose.

The reason why AC12 is redesigned into 2AC12 is that from the research on alpha-acarbose in binding with glucoamylase (PDB ID: 2QMJ), it was revealed that in the ligand-protein complex, the alpha-acarbose is bound to the protein active site primarily through side-chain interactions with its acarvosine unit, and almost no interactions were made with its glycone rings [15]. In the comparison of

the free binding energies of alpha-acarbose and acarvosine, the acarvosine structure achieved a lower free binding energy into both glucoamylase (-8.64 kcal/mol) and α-amylase (-7.01 kcal/mol). Corresponding to the comparison between AC12 and 2AC12, the 2AC12 compound also achieved a lower free binding energy than the four-ring AC12 structure, which is -11.12 kcal/mol in glucoamylase, and -8.99 kcal/mol in the α-amylase target protein. A more negative free binding energy indicates a stronger binding affinity and a more stable ligand-protein complex [16].

Looking at Figure 2D and 3C, there are attractive charges formed between the phosphate ion and the amino acid residues of the active binding pockets in both glucoamylase and α-amylase target protein. The amino acid residues in the glucoamylase binding pocket that have attractive charges were ASP443, ASP327, ASP571, ASP542 and PHE575, while for the α-amylase binding pocket, only one amino acid residue was involved, which was ASP300. The orange-dotted line of attractive charge bonds was linked directly to the phosphate ion of the 2AC12 compound (Fig. 2D & 3C). Attractive charges can be understood as a salt bridge interaction between groups of opposite charges in which at least one pair of heavy atoms is within the hydrogen bonding distance. This type of salt bridge interaction can contribute to stronger protein binding affinity; hence it can be observed that AC12 and 2AC12 which contained attractive charge interactions achieved a lower free binding energy than alpha-acarbose and acarvosine respectively (Table 1 & 2) [17].

When studying the binding interactions of 2AC12 to both 2QMJ and 4W93 generated using the Protein-Ligand Interaction Profiler (PLIP) web tool, to the binding interaction generated using BIOVIA Discovery Studio, there were some differences in the amino acid residues involved. From the results generated by PLIP, the 'attractive charge' term did not appear but was substituted with 'salt bridge.' This can be used to support that the attractive charge that occurred in between the ligand-protein complex is a type of salt bridge interaction, as mentioned above. The amino acid residue that was involved in the salt bridge interaction between 2AC12 and glucoamylase protein was HIS600. The other amino acids involved in hydrogen bonding and hydrophobic interactions were similar, just different in the addition of hydrogen bonding with ARG526 and ARG598, but with an absence of TRP406. Since the salt bridge was the strongest among all known noncovalent molecular interactions, this justified that 2AC12 achieved the lowest free binding energy (-11.12 kcal/mol) among all ligand compounds in the library that have docked into glucoamylase, hence having the strongest binding affinity [18].

From the binding analysis, 2AC12 compound which exhibited promising dual inhibitory activity, has been chosen as the potential dual inhibitor for the treatment of diabetes. To validate on the drug-likeness properties of the 2AC12 compound, further ADME analyses were carried out. Table 3 showed the absorption, distribution, metabolism, and excretion (ADME) of the 2AC12 compound which showed the strongest binding affinity to both glucoamylase and α-amylase as compared to the acarbose co-crystal ligand.

Table 3: Absorption, distribution, metabolism, and excretion (ADME) assessment of 2AC12 as the potential dual inhibitor for glucoamylase and α-amylase

Com-pound	MW	HBA: HBD	TPSA	Log P	Log S	GI absorption	BBB permeant	P-gp substrate	CYP inhibitor	Bioavai-lability
2AC12	401.3	12:9	219.21	-4.38	3.15	Low	No	Yes	0/5	0.11

Abbreviations: Molecular weight (MV, g/mol), number of hydrogen bond donor (HBD) and hydrogen bond acceptor (HBA), topological polar surface area (TPSA, Å), lipophilicity (Log P), solubility (Log S), gastrointestinal absorption (GI absorption), blood-brain barrier permeability (BBB permeant), P-glycoprotein substrate (P-gp substrate), and number of Cytochromes P450 inhibitor out of total 5 inhibitors (CYP inhibitor).

From the physicochemical and pharmacokinetic analysis of the 2AC12 compound by the ADME assessment, it can be suggested that the best route of administration for 2AC12 is through oral drugs in the form of a prodrug. The high lipophilicity and water solubility of 2AC12 make it suitable to be designed as an oral drug. However, to overcome the low GI absorption and low bioavailability score

(0.11) of the 2AC12 compound, designing 2AC12 into an oral drug in the form of a prodrug can help in improving a medication's effectiveness. This is because prodrug is originally in an inactive form, and will turn into an active form once they enter the body and reach the target site of action [19]. A prodrug can also be designed to avoid certain side effects or toxicities, which can be a great advantage for 2AC12 to be the potential treatment for diabetes.

Conclusion

Through virtual screening, and a series of molecular docking, a novel compound, 2AC12, was found to be a potential dual inhibitor of both glucoamylase (PDB ID: 2QMJ) and α-amylase (PDB ID: 4W93) target enzymes. This is because 2AC12 compound achieved the lowest free binding energy in both glucoamylase (-11.12 kcal/mol) and α-amylase (-8.99 kcal/mol), indicating that it was able to form stronger binding affinities with the target enzymes. The structure of 2AC12 was redesigned from the AC12 acarbose 7-phosphate compound (PubChem ID: 90659861), as research has shown that out of the four-ring structure of alpha-acarbose, only the two-ring acarvosine unit was responsible in the binding interactions in the enzyme binding pockets. The additional phosphate atom on 2AC12 was able to form salt bridge interactions with the amino acid residues on the binding pockets of both glucoamylase and α-amylase, makes it a better ligand with stronger binding affinity. From the ADME assessment of 2AC12, the best suggested route of administration for 2AC12 is through oral drug in the form of a prodrug. For future study, it is suggested that molecular dynamic simulations (MDS) can be carried out using the *Gromacs* software, to evaluate the binding stability of the ligand-protein complex.

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