

## Synthesis and Antidiabetic Evaluation of *N'*-Benzylidenebenzohydrazide Derivatives by *In Silico* Studies

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**Abstract:** Three new *N'*-benzylidenebenzohydrazide (NBB) derivatives were successfully synthesized and yielded 50–58%. FTIR, ESI-MS, <sup>1</sup>H- and <sup>13</sup>C-NMR were used to investigate the characteristic of NBB derivatives. The structure and relationship of NBB derivatives into  $\alpha$ -glucosidase and  $\alpha$ -amylase as good targets for diabetes treatment were evaluated using *in silico* screening. Molecular mechanics-Poisson Boltzmann/generalized born surface area (MM-PB/GBSA) was used to calculate the free binding energy ( $\Delta G_{\text{bind}}^{\text{MM-PB/GBSA}}$ ) of NBB to  $\alpha$ -glucosidase and  $\alpha$ -amylase receptors showed that the results of  $-0.45$  and  $-20.79$  kcal/mol, respectively. In the ortho position, NBB derivatives exhibited electron donating groups (EDG like  $-\text{OCH}_3$ ,  $-\text{OH}$  and  $-\text{Cl}$  with binding free energies of  $-21.94$ ,  $-6.71$ , and  $21.94$ , respectively, and acarbose, a native ligand energy of  $-32.62$  kcal/mol. In addition, the binding free energy of *N*-2-( $-\text{OCH}_3$ ,  $-\text{OH}$  and  $-\text{Cl}$ )-NBB to the  $\alpha$ -amylase receptor showed the number of  $-39.33$ ,  $-43.96$ ,  $-42.81$ , respectively and  $-46.51$  kcal/mol in comparing with a native ligand. As a result, it was found that all the NBB derivatives were able to interact with several amino acids in the  $\alpha$ -glucosidase cavity as well as the native ones, including Ala281, Asp282, and Asp616. NBB and native ligand showed similar interaction between  $\alpha$ -amylase with Gly110 amino acid residue.

**Keywords:** *N'*-benzylidenebenzohydrazide;  $\alpha$ -amylase; derivatives; antidiabetic; *in silico*

### ■ INTRODUCTION

Diabetes, in general, is a chronic metabolic disease characterized by elevated levels of blood glucose, which is divided into several types. Specifically, type-2 diabetes is a metabolic disorder distinguished by chronic hyperglycemia and either complete or partial deficiencies of insulin secretion [1]. Over the past few decades, there has been a rise in the prevalence of type-2 diabetes in many countries of the world from a wide range of income levels. An alternative therapeutic approach for controlling

hyperglycemia associated with type-2 diabetes is to target  $\alpha$ -glucosidase and  $\alpha$ -amylase enzymes that catalyze starch hydrolysis in the intestine. Inhibition of  $\alpha$ -glucosidase and  $\alpha$ -amylase can decrease hyperglycemia in non-insulin-dependent diabetes mellitus (NIDDM) and retard the absorption of glucose [2]. Acarbose, miglitol, and voglibose are the three  $\alpha$ -glucosidase inhibitors that have been approved for use in clinical trials at this time [3]. Voglibose comes from a microbial origin, whereas miglitol is synthetically derived

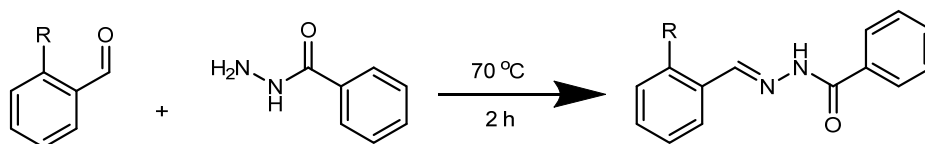


Fig 1. Synthesis of NBB and derivatives

from 1-deoxynojirimycin [4]. The  $\alpha$ -amylase is a calcium metalloenzyme that helps in the digestion of polysaccharide molecules into small saccharides. As same as  $\alpha$ -glucosidase, the  $\alpha$ -amylase enzyme causes postprandial hyperglycaemia and increased blood glucose levels. With these characteristics,  $\alpha$ -amylase is a well-known therapeutic target for the treatment and maintenance of elevated postprandial blood glucose [5].

*N'*-benzylidenebenzohydrazide (NBB) and its derivatives are important to the development of a significant class of new drugs [6]. Taha et al. [7] recently reported benzothiazole NBB derivatives containing benzohydrazide as  $\alpha$ -glucosidase inhibitor with a wide range of  $IC_{50}$  values. Ullah et al. [8] also reported that benzohydrazide-based imine and thiazolidine-4-one inhibit  $\alpha$ -glucosidase and  $\alpha$ -amylase enzymes. According to Fan et al. [9] chromone-based NBB derivatives may have the potential to perform as  $\alpha$ -glucosidase inhibitors. The core of benzohydrazide is crucial to the inhibition of their enzymes. Therefore, the research aims to synthesize and evaluate NBB-derived with electron-donating groups ( $-OCH_3$ ,  $-OH$  and  $-Cl$ ) (Fig. 1) as antidiabetic to target  $\alpha$ -glucosidase and  $\alpha$ -amylase enzymes by using molecular docking.

## ■ EXPERIMENTAL SECTION

### Materials

Analytical grades of benzohydrazide, benzaldehyde, salicylaldehyde, *o*-anisaldehyde, and *o*-chlorobenzaldehyde were purchased from Sigma Aldrich. Analytical grades of hexane, dimethylformamide, dimethyl sulfoxide, dichloromethane, sodium acetate and glacial acid were purchased from Merck. The pure solvents of ethanol 99.9%, methanol 99.8%, chloroform, and ethyl acetate were prepared from Fulltime.

### Instrumentation

NMR measurement was used TMS as an internal reference, NMR spectra were recorded on a JEOL

Resonance 400 MHz spectrometer, and the chemical shifts were reported in  $\delta$  (ppm). TLC was performed using silica gel 60 F254 aluminium sheet, while ESI-MS data were obtained using water mass spectrometer Q-TOF XEVO. At last, functional groups were analyzed using FTIR Bruker Opus with KBr pellet preparation. Software for docking analysis: the computing study in this research was performed under a Dell WorkStation Personal Computer, Linux Ubuntu 20.04.3 LTS OS, Intel® Xeon(R) W-2223 CPU @3.60 GHz octa-core; RAM 16 GB and GPU NVIDIA Quadro P2200. Meanwhile, molecular docking was conducted with Maestro Schrödinger 2022-1 software (Schrödinger, New York, NY, USA).

### Procedure

#### Synthesis of NBB derivatives

NBB synthesis was conducted by using the reflux process and several modified procedures from Jubie et al. [10]. The ligands were prepared by adding 6 mmol (0.8169 g) of benzohydrazide in 30 mL of ethanol. Then, 6 mmol of the *o*-benzaldehyde derivative and 30 mL of ethanol were added to the flask with a small amount of acetic acid. The mixture was refluxed for 2 h at 70 °C temperature. After that, the product was cooled overnight at 4 °C and separated by using a funnel. As the last step, an aluminium sheet with TLC gel 60 F<sub>254</sub> was used for product tracing. This procedure was repeated for the synthesis of the other four derivatives, such as *N*-2-(-Cl, -OH, and -OMe)benzohydrazide.

***N'*-(2-chlorobenzylidene)-benzohydrazide 1.** Yield: 53.40%. FTIR (KBr,  $cm^{-1}$ ): 3181 ( $-C_{sp^2}H$  aromatic); 1643 ( $-C=O$ ); 1555 ( $-N=N-$ ).  $^1H$ -NMR (DMSO- $d_6$ ): 12.05 (s, 1H,  $-NH$ ); 8.84 (s, 1H,  $-CH=N$ ); 8.00 (m, 1H); 7.91 (d, 2H); 7.57 (t, 1H); 7.50 (dd, 3H); 7.42 (m, 2H).  $^{13}C$ -NMR (DMSO- $d_6$ ): 163.8 ( $-C=O$ ); 144.2 ( $-CH=N$ ); 133.8; 133.7; 132.5; 132.1; 132.1; 130.5; 129.1; 128.2; 128.2; 127.4. ESI-MS: 259.0636 (100%)  $[L+H]^+$ ; 281.0360 (100%)  $[L+Na]^+$ .

***N'*-(2-hydroxybenzylidene)-benzohydrazide 2.** Yield: 50.12%. FTIR (KBr,  $\text{cm}^{-1}$ ): 3268 (-NH); 3268 (-OH); 3057 ( $-\text{C}_{\text{sp}^2}\text{H}$  aromatic); 1672 (-C=O); 1538 (-N=N); 1271 (-C-O).  $^1\text{H-NMR}$  (DMSO- $d_6$ ): 12.1 (s, 1H, -NH); 11.37 (s, 1H, -CH=N); 8.61 (s, 1H); 7.90 (m, 2H); 7.58 (t, 1H); 7.51 (ddd, 3H); 7.27 (td, 1H); 6.90 (m, 2H).  $^{13}\text{C-NMR}$  (DMSO- $d_6$ ): 163.4 (-C=O); 158.0 (-C-OH); 148.9 (-CH=N-); 133.4; 132.5; 132.1 130.1; 129.1; 128.2; 119.9; 119.2; 117.0. ESI-MS: 241.0979 (100%) [L+H] $^+$ .

***N'*-(2-methoxybenzylidene)-benzohydrazide 3.** Yield: 57.93%. FTIR (KBr,  $\text{cm}^{-1}$ ) 3184 ( $-\text{C}_{\text{sp}^2}\text{H}$  aromatic); 2988 ( $-\text{C}_{\text{sp}^3}\text{H}$  of methyl); 1640 (C=O); 1556 (-N=N); 1251 (-C-O).  $^1\text{H-NMR}$  (DMSO- $d_6$ ): 11.81 (s, 1H, -NH); 8.79 (s, 1H, -CH=N); 7.89 (m, 2H); 7.85 (dd, 1H); 7.55 (dd, 1H); 7.48 (t, 2H), 7.38 (m, 1H); 7.07 (d, 1H); 6.99 (t, 1H); 3.83 (s, 3H,  $-\text{OCH}_3$ ).  $^{13}\text{C-NMR}$  (DMSO- $d_6$ ): 163.5 (-C=O); 158.3 (-C=OCH $_3$ ); 143.8 (-CH=N); 133.9; 132.3; 132.1; 129.0; 128.2; 126.1; 122.9; 121.3; 112.4; 56.2 ( $-\text{OCH}_3$ ). ESI-MS: 255.1136 (100%) [L+H] $^+$ .

### Ligands and receptor preparation

ChemDraw was used to generate the ligand structures, which were then converted into a 3D model using LigPrep [11] module in Schrodinger 2022-1 as well as protonated at pH 7.4 with Epik [12] and OPLS4 forcefield [13]. These processes aim to restore improper or missing bonds, assign protonation, possible ionization, and tautomeric states [14-16]. Moreover,  $\alpha$ -glucosidase (PDB ID: 5NN8) protein was prepared by removing the residual solvent, optimizing the hydrogen bond, and protonating using ProtAssign [17] and PROPKA [18]. At the same time, in the "protein preparation wizard" that is incorporated into Maestro Schrodinger 2022-1 [19-20], the partial charge was also added using OPLS4 forcefield.

### Molecular docking

The docking study was performed using Glide [21] Maestro Schrodinger's 2022-1 to predict the binding affinities and molecular interactions of synthesized compounds against two receptor targets:  $\alpha$ -glucosidase (PDB ID: 5NN8) and  $\alpha$ -amylase (PDB ID: 6GXV). Acarbose, an inhibitor that acts as the native ligand, was used for comparing to compounds. A grid box was placed at the center of acarbose position with similar dimensions

for both receptors ( $20 \times 20 \times 20 \text{ \AA}$ ) for setting the docking region. The docking grid box for  $\alpha$ -glucosidase and  $\alpha$ -amylase were assigned based on the coordinates of the native ligand at ( $x = -13.92, y = -38.29, z = 95.23$ ) and ( $x = 44.03, y = 22.72, \text{ and } z = -11.87$ ), respectively. Redocking the native ligand was conducted to validate docking protocols and calculated their root mean square deviation (RMSD) with 100 conformations limit numbers. The docking protocol was deemed valid if the RMSD value was  $< 2 \text{ \AA}$  [22]. The docking process was carried out with Glide in extra precision (XP) mode under rigid receptors and flexible ligand conditions. The molecular mechanics-generalized Born surface area (MM-GBSA) was calculated to assess the docking pose of the ligands and determine the potency of each compound [23-26]. The "ligand interactions panel" on Maestro Schrodinger was used to visualize the molecular interactions. The results provide valuable insights into the binding modes and mechanisms of these receptors. It could help to guide the design of new compounds with improved efficacy and specificity.

## RESULTS AND DISCUSSION

### Computer-Aided Drug Discovery

Computer-aided drug discovery is the most important tool to predict drug activity through computational structure-based drug discovery. The relationship between sites of protein action and compounds acting as ligands can be explained using a variety of software. In point of fact, a physics-based equation is used to determine the binding free energy [27]. The  $\alpha$ -glucosidase is an essential enzyme that is found on the luminal surface of enterocytes that functions to regulate blood glucose by converting complex carbohydrates into absorbable glucose, which is required for energy metabolism [28-29]. The removal of the anomeric carbon from the glucosyl group and the glycosidic oxygen ( $\text{C}_1\text{-O}$ ) is the first step in the hydrolysis reactions carried out by  $\alpha$ -glucosidases. Then, the glucosyl group is replaced by a proton from water resulting in the process of hydrolysis and transglycosylation exchange process between glucosyl residues and the protons [30]. Acarbose (Fig. 2) inhibits

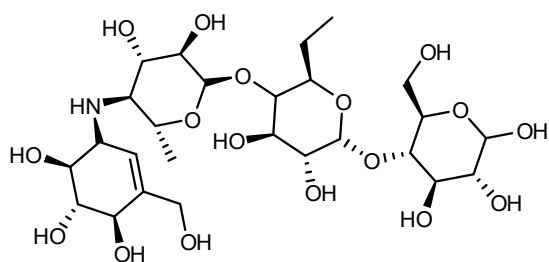


Fig 2. Acarbose

intestinal  $\alpha$ -glucosidases, the enzymes responsible for the metabolism of complex carbohydrates into absorbable monosaccharide units in a reversible manner. This mechanism might be used to identify and develop new diabetic medications that are useful for showing diabetes progression [31-34].

### Molecular Docking

Molecular docking is an important and helpful tool to predict the binding affinity of molecules to proteins [6]. In this study, the docking protocol was validated by internal validation after redocking the native ligand in its original positions resulting in RMSD values of 1.812 and 1.165 Å for  $\alpha$ -glucosidase and  $\alpha$ -amylase receptors. Based on these findings, it is possible to determine the test compound's activity against  $\alpha$ -glucosidase as well as  $\alpha$ -amylase receptors using either docking protocol [22]. Prior to docking the molecule, RMSD was used to determine the native acarbose ligand's docking position, as depicted in Fig. 3.

Compounds NBB 1, 2, and 3 with aromatic parts in their structures were evaluated for the protein-ligand

complexes in the structure-activity relationship. It was discovered that a more negative score of binding affinity indicated a stronger binding. This ligand-protein binding process is correlated with this score which is also known as the change of the free energy. The measurement of how strong the interaction between the ligand and the protein is often directly related to the potential for ligand activity [35-36]. NBB without substituents resulted in an MMGBSA score of  $-0.45$  on the binding affinity of complexes ligand-receptors. The *ortho* positions of the NBB derivatives with EDG ( $-Cl$ ,  $-OH$  and  $-OMe$ ) were  $-18.28$ ,  $-6.71$ , and  $-21.94$  kcal/mol, respectively. As can be seen in Table 1 and Fig. 4, the results of the ligand-receptor binding indicated that these compounds were bound to the same residues (Ala284, Asp282 and Asp616) in the entry area of the  $\alpha$ -glucosidase active site.

The native acarbose inhibitor was validated by a redocking process on  $\alpha$ -amylase in its original positions, which resulted in an RMSD value of 1.165 Å. In Table 2, the docking result between NBB derivatives and the  $\alpha$ -amylase receptors showed that the value of MMGBSA as a binding affinity score for the ligand-receptors complex had a nearly equal range between  $-43.09$  to  $-39.42$ , with native ligand acarbose  $-46.51$  kcal/mol. Similar to  $\alpha$ -glucosidase, the NBB grid score,  $-20.78$  kcal/mol, was found to be greater than the NBB derivatives. Through the use of a hydrogen bond, NBB derivatives were able to interact with the same amino acids in the cavity of  $\alpha$ -amylase Gly110 in a strong  $\pi$ - $\pi$  interaction [37]. It should

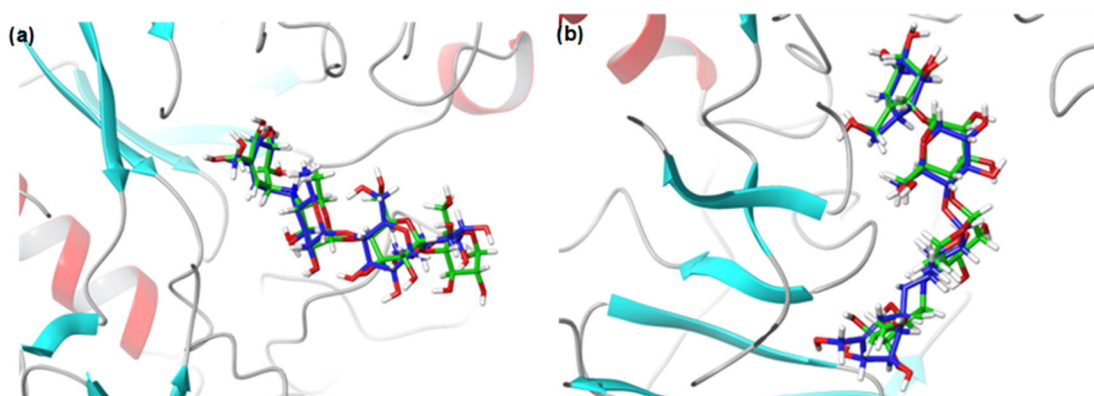
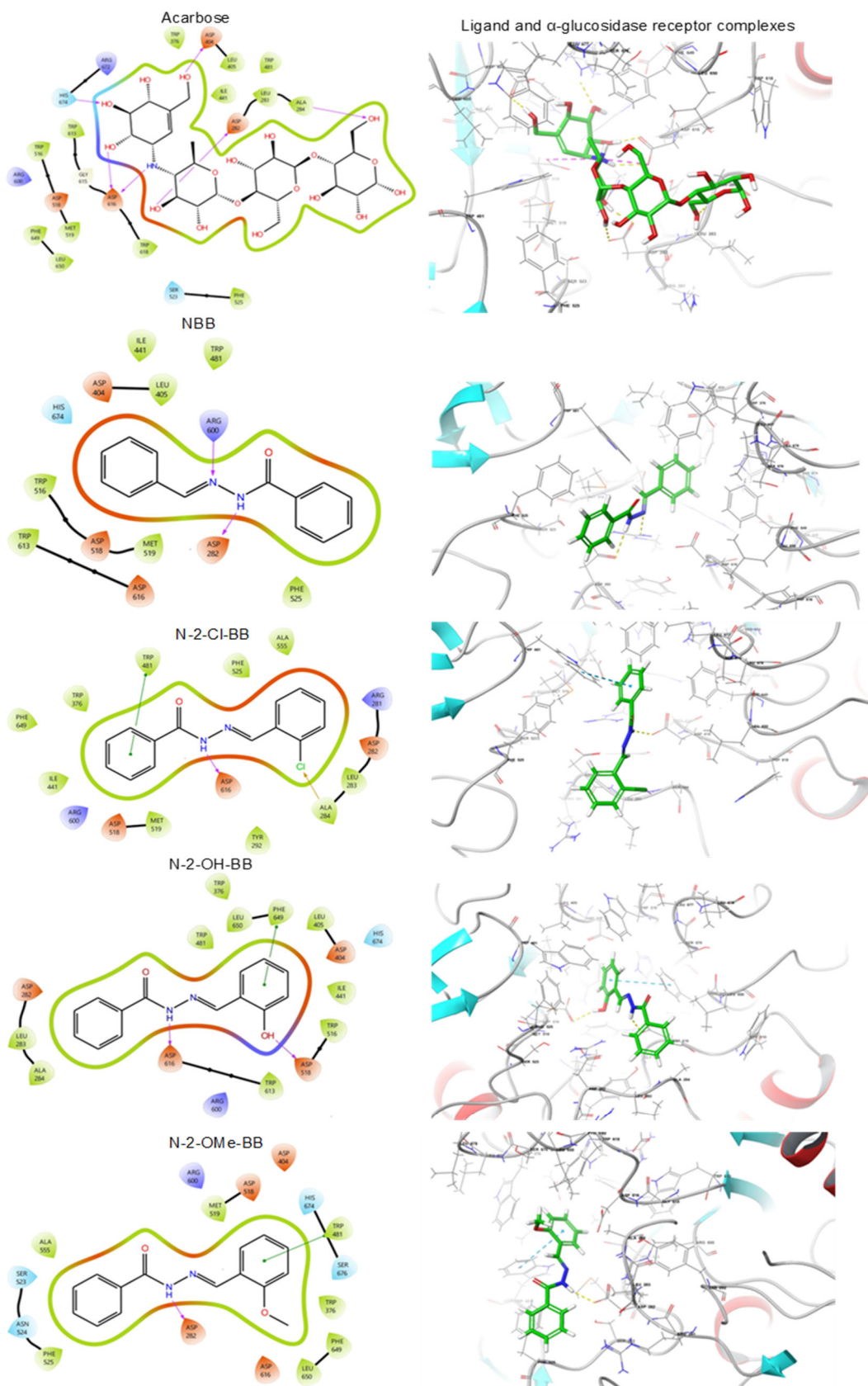


Fig 3. The acarbose native ligand on (a)  $\alpha$ -glucosidase and (b)  $\alpha$ -amylase receptors (Blue = original position and green = native ligand after redocking)



**Table 1.** Molecular docking result against  $\alpha$ -glucosidase receptor

No.	Compound	MMGBSA (kcal/mol)	Amino acid interaction
1	<i>N</i> '-benzylidenebenzohydrazide	-0.45479	<b>Asp282</b> , Arg600
2	<i>N</i> '-(2-chlorobenzylidene)benzohydrazide, <b>1</b>	-17.2886	<b>Ala284</b> , Trp481, <b>Asp616</b>
3	<i>N</i> '-(2-hydroxybenzylidene)benzohydrazide, <b>2</b>	-6.71190	Asp518, <b>Asp616</b> , Phe649
4	<i>N</i> '-(2-methoxybenzylidene)benzohydrazide, <b>3</b>	-21.9442	<b>Asp282</b> , Trp481
5	Acarbose (Native ligand)	-32.6238	<b>Asp 282</b> , <b>Ala284</b> , Asp404, <b>Asp616</b> , His674

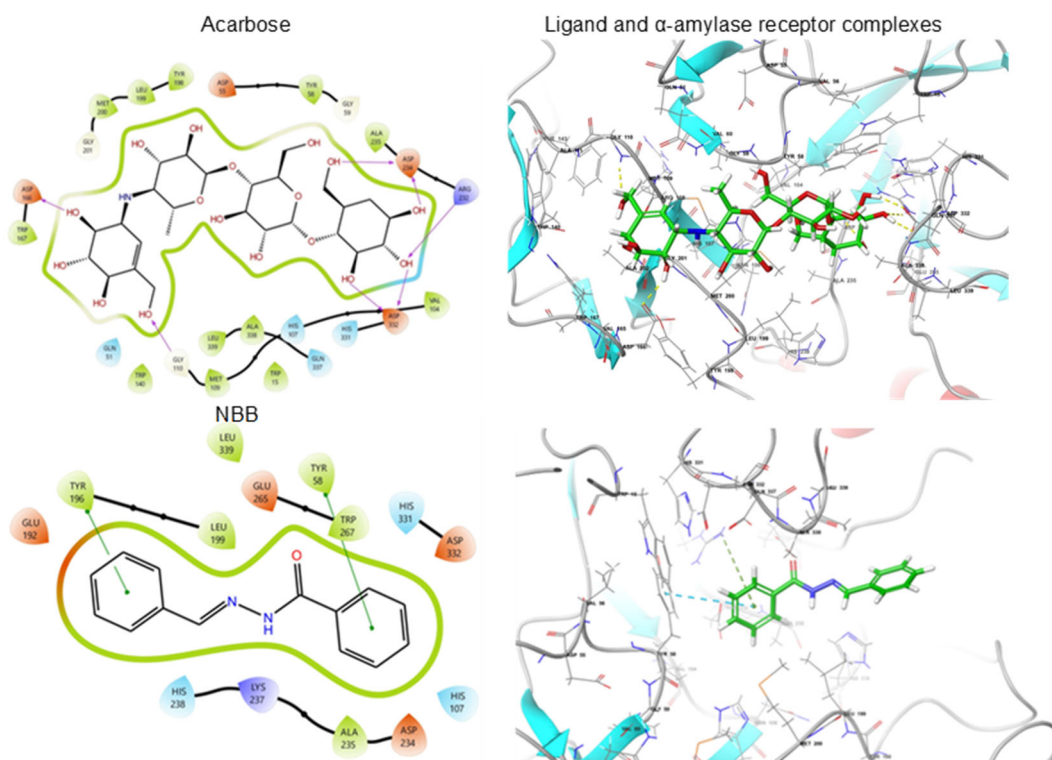
**Table 2.** Molecular docking results against  $\alpha$ -amylase receptor

No.	Compound	MMGBSA (kcal/mol)	Amino acids interaction
1	Acarbose (Native ligand)	-46.5148	<b>Gly110</b> , Asp166, Asp234, Arg232, Asp332
2	<i>N</i> '-benzylidenebenzohydrazide	-20.7892	Tyr58, Tyr196
3	<i>N</i> '-(2-chlorobenzylidene)benzohydrazide, <b>1</b>	-42.8137	<b>Gly110</b> , Ala111
4	<i>N</i> '-(2-hydroxybenzylidene)benzohydrazide, <b>2</b>	-43.0944	<b>Gly110</b> , Gln51
5	<i>N</i> '-(2-methoxybenzylidene)benzohydrazide, <b>3</b>	-39.3278	<b>Gly110</b> , Ala111

be noted that NBB was not involved in binding interactions with Gly110 and other amino acid residues that include the native ligand (Table 2).

Through a variety of hydrophobic and hydrogen bonds, the active site of residue  $\alpha$ -amylase receptor with NBB and its derivatives can be followed in Fig. 5.

The compounds synthesized exhibit affinity energy values that are suboptimal compared to native ligands. However, some of the synthesized compounds show interactions with the catalytic site of the  $\alpha$ -glucosidase receptor, specifically *N*'-(2-chlorobenzylidene)benzohydrazide, *N*'-(2-hydroxybenzylidene)benzohydrazide,



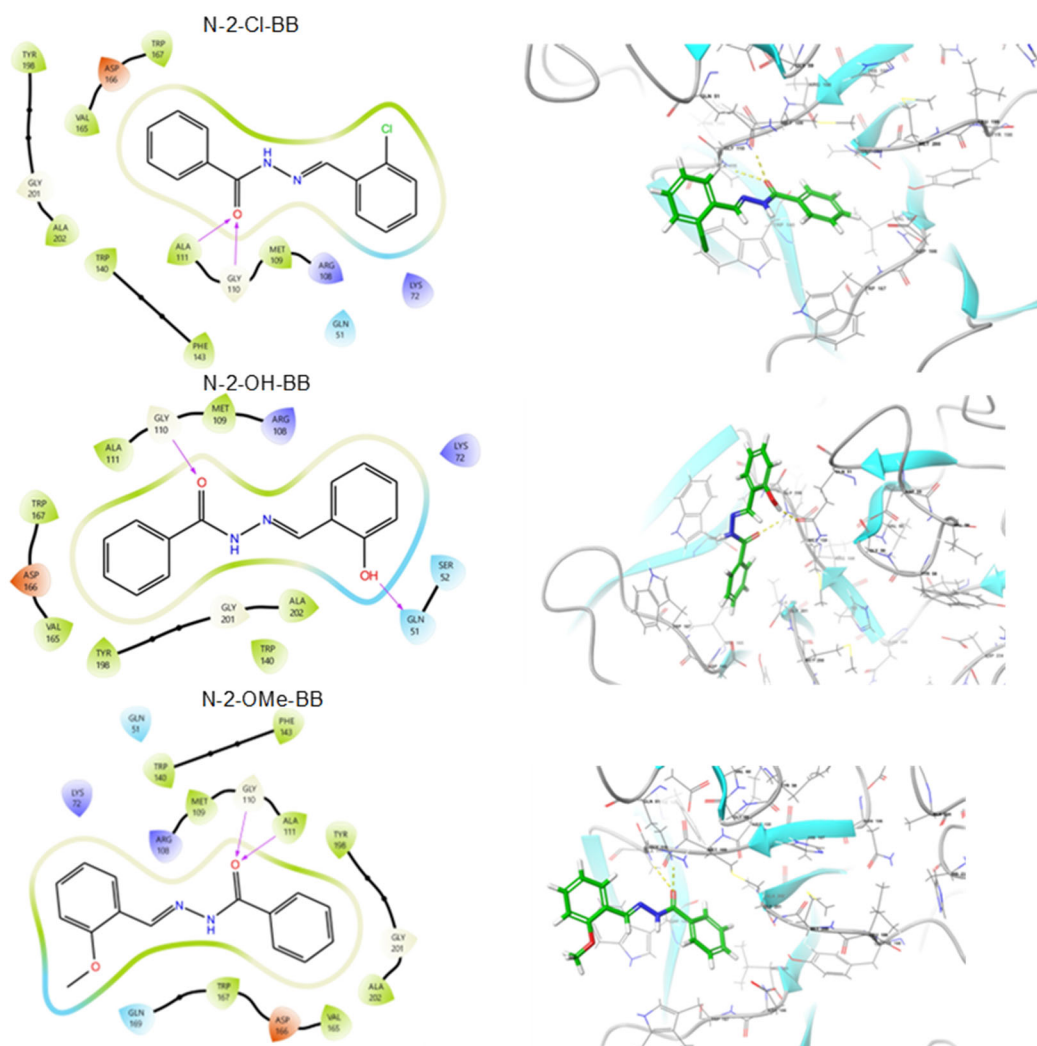


Fig 5. The binding of NBB and derivatives toward  $\alpha$ -amylase receptor

and *N'*-(3-nitrobenzylidene)benzohydrazide on amino acids Asp518 and Asp616. Although these interactions can inhibit the activity of the  $\alpha$ -glucosidase enzyme, they do not perform as well as the native ligand due to the lack of other molecular interactions that could enhance the affinity of the interaction. Despite this limitation, these three compounds hold the potential for further development as  $\alpha$ -glucosidase inhibitor agents [38].

## ■ CONCLUSION

In drug design, the molecular docking technique has been used extensively to predict the ligands-receptor interactions. Substituents on the benzene ring play a role in antidiabetic activity. NBB derivatives ligand with

electron-donating groups at the *ortho* position has the potential to increase the activity of antidiabetic target receptors for both  $\alpha$ -glucosidase and  $\alpha$ -amylase. Numerous functional groups and para positions will be performed in the subsequent experiment.

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## ■ AUTHOR CONTRIBUTIONS

Yusuf Syarif Alam, Nur Rahmayanti Afifah, Arif Fadlan conducted the synthesis experiment, Tutik Sri Wahyuni and Saipul Maulana performed docking analysis. Pratiwi Pudjiastuti, Fahimah Martak and Arif Syukri wrote and revised the manuscript. All authors agreed to the final version of this manuscript.

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