Docking-based virtual screening and pharmacophore analysis of novel GH-20 β-N-acetylglucosaminidase inhibitors

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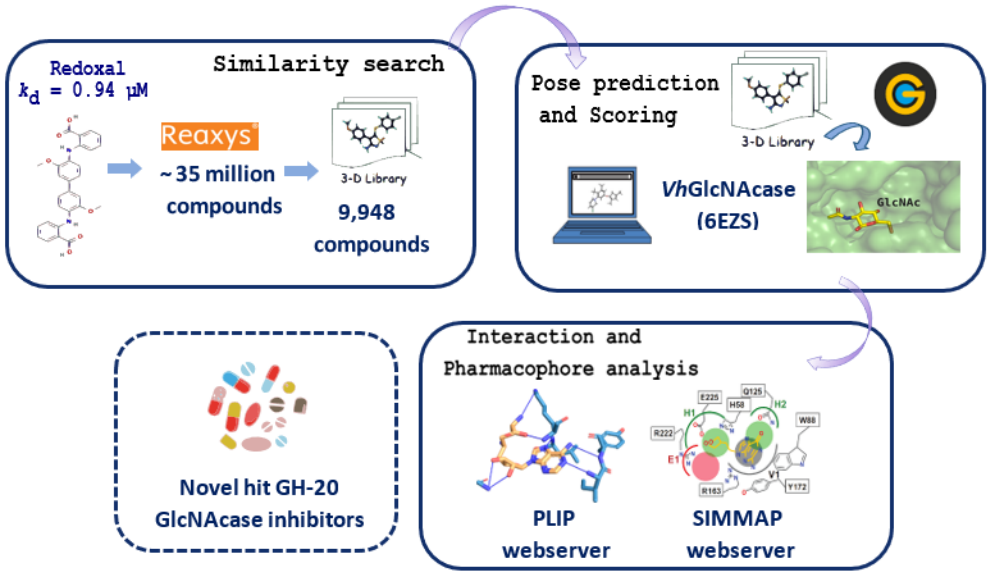
Abstract: GH-20 β-N-Acetylglucosaminidases (GlcNAcase) is a promising target for the development of new drugs active against *Vibrio* infections since the suppression of GH-20 GlcNAcase activity can lead to the inhibition of *Vibrio* spp. growth. In this study, we set up a virtual screening for potential GH-20 GlcNAcase inhibitors from the Reaxys commercial database, using a known active compound (Redoxal) as a ligand search model and GlcNAcase from *V. harveyi* (*Vh*GlcNAcase) as a protein search model. Virtual screening results identified the top ten compounds, with ChemPLP scores between 98.27 and 104.15. All ten compounds had scores greater than those of Redoxal (85.74) and the natural substrate $(GlcNAc)_4$ (85.40). Interactions and pharmacophore analysis indicated that W582 is a key residue that interacts with all the identified molecules, mainly by π-stackings interactions. The inner part of all ten molecules is located at subsite -1, deep in the catalytic pocket of *Vh*GlcNAcase, while the outer part stretches beyond the binding pocket. Hit compounds identified in this study may serve as potential candidates for further development of new, highly potent antimicrobial agents for controlling *Vibrio* spp. infections in both aquaculture and humans.

Graphical abstract:



Keywords: Virtual screening; Molecular docking; β-N-acetylglucosaminidases; *Vh*GlcNAcase; GH-20 GlcNAcases inhibitors; Protein-drug interactions

1. Introduction

Vibrios are Gram-negative bacteria, found as ubiquitous inhabitants of marine and aquatic ecosystems worldwide as well as in aquaculture farms, and are major microbiota in these ecosystems. Nearly 100 *Vibrio* species have now been identified. Many are serious pathogens for animals grown in aquaculture and around 12 species can cause infections in humans [1]. Vibriosis, infection by *Vibrio* spp., is one of the most common diseases in fish and other aquaculture-reared organisms and is widely responsible for fatalities in cultured aquaculture systems worldwide, which devastates seafood-based economies [2,3]. Several approaches to controlling Vibrio-prone diseases in aquaculture have been made, including the use of antibiotics, however, the rate of infection remains alarming in many environments due to factors such as antibiotic resistance. Disease maintenance with antibiotics demonstrated against many ranges of bacteria including *Vibrio* spp., leading to its widespread in aquatic systems. Antimicrobial agents such as tetracycline, fluoroquinolones, and azithromycin have been successfully used for the treatment of cholerae disease (i.e., patient infection with *V. cholerae*) [4]. However, in recent years, treatment failure has been widely encountered, with the emergence of strains of *V. cholerae* displaying antimicrobial multidrug resistance (AMR) [5].

Vibrio spp. live mostly in marine and aquatic saltwater, which lacks glucose enrichment. However, these bacteria are viable because of their evolutionary adaptation to utilizing chitin, a GlcNAc-linked polymer that is highly abundant in oceans, as a nutrient. Since marine *Vibrio* spp. are dependent on the availability of chitin in marine ecosystems, the activity of chitin-degrading enzymes and chitin-binding proteins in the chitin degradation pathway is necessary for the growth of these bacteria. Two major groups of glycoside hydrolases are found in *Vibrio* bacteria: endo-chitinases (EC 3.2.1.14) [6] and exo-β-N acetylglucosaminidases or GlcNAcases (EC 3.2.1.52) [7]. GlcNAcases are usually found in the periplasm, where they participate in the chitin catabolic cascade by sequentially degrading chitin oligosaccharide fragments, with β-N-Acetylglucosamine (GlcNAc) as the final product. The GlcNAc monomers then enter the GlcNAc/GlcN metabolic pathway, which enables *Vibrio* bacteria to use the chitin catabolic product (GlcNAc) as their sole source of carbon, nitrogen, and energy for cellular growth [6,7]. Thus, GlcNAcases are excellent candidates for the development of new inhibitors to be used as antimicrobial agents directed against *Vibrio* infection. In the CAZy database (http://www.cazy.org/), GlcNAcases are mainly divided into three glycoside hydrolase families: GH-3, GH-20, and GH-84. All GlcNAcases found in bacteria are classified in the GH-20 glycoside hydrolase family. *Vh*GlcNAcase is one of the GH-20 β-N-Acetylglucosaminidases found in *V. harveyi*. It acts as an exo-acting enzyme that degrades chitooligosaccharides from the non-reducing end in a sequential manner, generating GlcNAc as the final product.

Numerous efforts have been reported in the search for novel GH-20 GlcNAcases inhibitors. For example, PUGNAc (N-Acetylglucosaminono-1,5-lactone O-(phenylcarbamoyl)oxime) has been characterized as a potent inhibitor of GlcNAcases not only from GH20 [8] but also GH3 [9] and GH84 [10]. NAG-thiazoline (N-Acetyl-Glucosamine thiazoline), shown to be a potent inhibitor of both GH20 [11] and GH84 GlcNAcases [8], and iminosugars such as 2-acetamido-1,2 dideoxynojirimycin (NHAcDNJ) and 6-acetamido-6-deoxy-castanospermine (NHAcCAS) have also been found to be the potent inhibitors of GlcNAcases from GH3, GH20, and GH84 [12]. Meekrathok et al. successfully performed virtual screening and identified novel inhibitors, using *Vh*GlcNAcase as a search model to screen the anti-cancer compounds available in the National Cancer Institute (NCI) database. The most potent GlcNAcase inhibitor identified in their study is Redoxal, which has IC_{50} = 12.7 ± 1.2 μM and K_d = 0.94 ± 0.2 μM [13].

Traditional drug development is notoriously time-consuming and cost-intensive, lead compound identification being one of the most important and difficult steps in modern drug design and discovery. Thus, numerous methods and strategies have been developed and used to identify promising lead candidates for a target of interest. Among them, Structure-Based Virtual Screening (SBVS) has become an essential tool in assisting fast and cost-efficient lead discovery and optimization. Recently, new biologically active compounds have been predicted along with their receptor-bound structures and in several cases the achieved hit rates (ligands discovered per molecules tested) are significantly greater than when a high-throughput screening approach alone was used [14-16].

In our study, we set up virtual screening for identified novel GH-20 GlcNAcase inhibitors using a scoring function embedded in GOLD software to rank compounds from the Reaxys commercial substances database using a known active compound, Redoxal, as a ligand search model and *V. harveyi* GH-20 GlcNAcase (*Vh*GlcNAcase) as a protein search model. The novel GlcNAcase inhibitors identified in this study could be used for further identification of new active compounds that act as potent GH-20 GlcNAcase inhibitors.

2. Materials and Methods

2.1. Preparation of the protein structure

The X-ray crystal structure of *Vh*GlcNAcase complexed with N-Acetylglucosamine (GlcNAc) was initially selected as the search target in virtual screening. The X-ray crystal structure was obtained from the Protein Data Bank (accession number: 6EZS) with 2.5 Å resolution. For docking with GOLD, hydrogen atoms were introduced into the *Vh*GlcNAcase molecule using the ionization and tautomeric states inferred by the GOLD program [18]. All water molecules, ions, and ligands were then removed from the crystal structure.

2.2. Re-docking of the GlcNAc into the VhGlcNAcase binding site

GlcNAc was re-docked into the sugar-binding sites of *Vh*GlcNAcase (PDB ID: 6EZS). The GlcNAc structure was downloaded from Pubchem (https://pubchem.ncbi.nlm.nlh.gov/) and its geometries were optimized to relaxed forms. GlcNAc was then re-docked and scored by the scoring function embedded in the GOLD software. The score value and Root Mean Square Deviation (R.M.S.D.) between docked-pose and crystal-pose were evaluated for further screening.

2.3. Ligand library selection and preparation

The structure of the known active compound (Redoxal) was downloaded from Pubchem and was used as input in Reaxys websites for similar searching within a commercial substance database available at www.reaxys.com. Reaxys commercial substances is a fully integrated supplier database of a growing pool of over 330 aggregated chemical vendors (including the aggregators eMolecules and LabNetwork) containing over 35 million unique molecules and 95 million associated products, including screening compounds and building blocks. All Redoxal-similar compound structures were taken in sdf MDL MOL format from these websites. The dataset contains 9,948 2D chemical structures of compounds similar to Redoxal. Their chemical structures were generated with 3D atomic coordinates, cleaned, hydrogen atoms added, and ligand minimization performed using the RDkit package [19].

2.4. Virtual screening and molecular docking

All compounds were virtually screened using the molecular docking algorithms available in GOLD v5.3.0, an algorithm-based program that performs fast and flexible ligand docking [18]. Chemical structures of 9,948 compounds were docked into the 6EZS

crystal structure and scored using the ChemPLP scoring function. The automatic genetic algorithm (GA) parameter setting was used in all the GOLD docking calculations. A hundred percent search efficiency was applied, with a minimum of 10,000 and a maximum of 125,000 operations per ligand. The number of GA runs was set to 150. To define the key inhibitor-binding sites, only amino acid residues of *Vh*GlcNAcase within a 15 Å radius of the center of the bound GlcNAc were considered. Other parameters were set by default. The 150 diverse solutions were generated, the docking scores ranked by GOLD and the top ten hits from the GOLD ranking were initially selected for visual interaction inspection and further enzyme-ligand pharmacophore analysis.

2.5. Interaction and pharmacophore analysis

Virtual docking of the compounds in the substrate-binding pocket of the enzyme was achieved and protein-ligand interactions of the ten highest-scoring compounds were then evaluated. The Protein-Ligand Interaction Profiler (PLIP) [20] was used to calculate the interactions of *Vh*GlcNAcase with the hit compounds. Finally, all poses generated for the top ten compounds from GOLD were used in protein-ligand pharmacophore analysis. Interaction preferences of the functional groups of the top ten hit compounds were analyzed using the SiMMap server (http://simmap.life.nctu.edu.tw/) [21]. A three-dimensional diagram generated from PLIP and SiMMap was automatically visualized and generated using the program Pymol [22]. The virtual screening workflow used in this study is summarized in Figure 1.

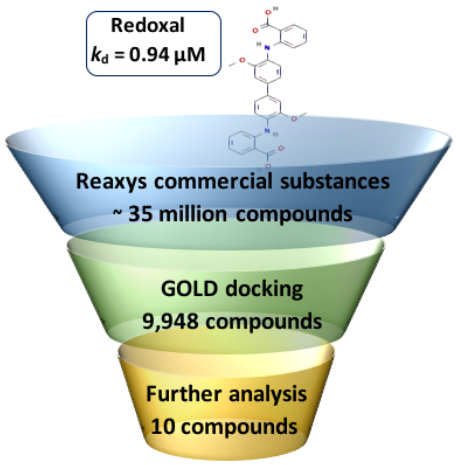


Figure 1. Workflow of docking-based virtual screening in this study

3. Results

3.1. Re-docking of GlcNAc into the VhGlcNAcase substrat- binding sites

Re-docking GlcNAc into the *Vh*GlcNAcase (PDB id 6EZS) binding pocket yielded the highest ChemPLP score of 58.41±0.12 when compared with other scoring functions, along with R.M.S.D. less than 2 Å (1.01±0.01) when compared with crystal pose (Table 1). Thus, ChemPLP was used as a scoring function to rank the efficacy of the screened compounds during the virtual screening step.

Table 1. Re-docking N-acetylglucosamine (GlcNAc) into the 6EZS binding pocket

Scoring function	Score	R.M.S.D.
ChemPLP	58.41±0.12	1.01±0.01
ChemScore	20.15±0.69	0.46±0.05
GOLDScore	54.71±0.21	5.20±0.02
ASP	42.56±0.40	5.26±0.03

3.2. Virtual screening and molecular docking

In a search for novel GlcNAcase inhibitors, we carried out virtual screening of Redoxal-like compounds from the Reaxys commercial substance database. Initial hits identified 9,948 compounds, which were further docked into the crystal structure of *Vh*GlcNAcase using N-Acetylglucosamine (GlcNAc) as a reference ligand. Screening finally identified ten hits that had Piecewise Linear Potential (PLP) scores between 98.27 and 104.15. These scores were greater than those of the reference compound Redoxal (ChemPLP score = 85.74) and the natural substrate $GlcNAc_4$ (ChemPLP score = 85.40). Of these, compound 338175 showed the highest PLP score of 104.15, followed by 1146525 (101.66) and 340469 (100.25) (Table 2.). The chemical structures of the top ten hit compounds are shown in Figure 2.

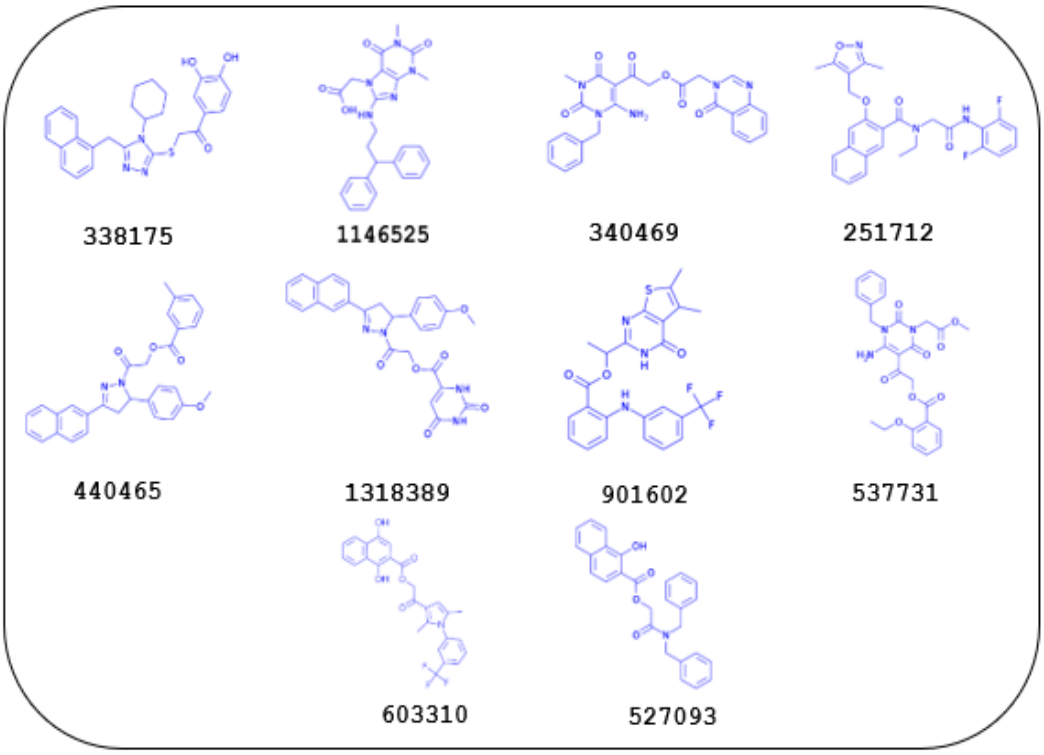


Figure 2. 2D chemical structures of the top ten compounds from the ChemPLP scores.

Table 2. Top ten compounds identified from ChemPLP scores after virtual screening compared with the natural substrate $(GlcNAc)_4$ and Redoxal as reference compounds.

No.	Compounds	ChemPLP score	M.W. (g/mol)	LogP	No. of H-bond donor	No. of H-bond acceptor	No. of rotatable bond
	$(GlcNAc)_4$	85.40	884.8	-	-	-	-
	Redoxal	85.74	484.5	6.7	4	8	9
1.	338175	104.15	473.6	7.4	2	4	7
2.	1146525	101.66	447.5	3.1	2	9	8
3.	340469	100.25	475.5	1.0	1	11	8
4.	251712	100.21	493.5	5.0	1	4	10
5.	440465	99.59	478.5	6.8	0	5	8
6.	1318389	99.55	498.5	3.5	2	9	8
7.	901602	99.52	487.5	5.1	2	6	7
8.	537731	98.55	495.5	2.5	1	10	12
9.	603310	98.55	483.4	6.2	2	4	7
10.	527093	98.27	425.5	5.9	4	1	9
	GlcNAc	59.30	221.2	-1.7	5	6	2

3.3. Interaction and pharmacophore analysis

From interaction analysis, most of the compounds show π-stacking interactions (shown by green dashed lines) between aromatic rings in the compound and tryptophan or tyrosine sidechains (W505, W546, W582, Y46 and Y530), which create a strong hydrophobic environment in the substrate-binding pocket of *Vh*GlcNAcases; in particular, the inner part of all the identified compounds formed π-stacking interactions with W582. In addition, hydrogen bonds were found in many complexes, especially with the residues R45 and R274. The interaction of the top three compounds (338175, 1143525 and 340469) and the crystal pose of GlcNAc within the *Vh*GlcNAcase binding pocket are shown in Figure 3. SiMMap was used to further evaluate the preferred interaction between the enzyme's substrate-binding pocket and the compounds. Pharmacophore analysis demonstrated that the inner half of all top ten compounds from the ChemPLP entity is located toward the bottom of the catalytic cavity at subsite -1 (referred to as V1 anchor). The highly conserved residue W582 makes interactions with the core cyclic structures of all of the identified compounds, with a preference for the aromatic ring (54%), followed by the phenolic ring (17%) and heterocyclic ring (9%), while the outer half of the compound stretches towards the surface of the active site (V3 anchor) (Figure 4a). Superimposition of the top three compounds clearly shows that the inner ring of the compound locates at V1 anchor and stacks against the W582 residue (Figure 4b). It is clear that the key binding residue W582 in *Vh*GlcNAcase (W278 in hOGA, W460 in HexA and W489 in HexB) is completely conserved within the GlcNAcase family, and pharmacological analysis suggested that this residue may be essential for enzyme-drug interactions.

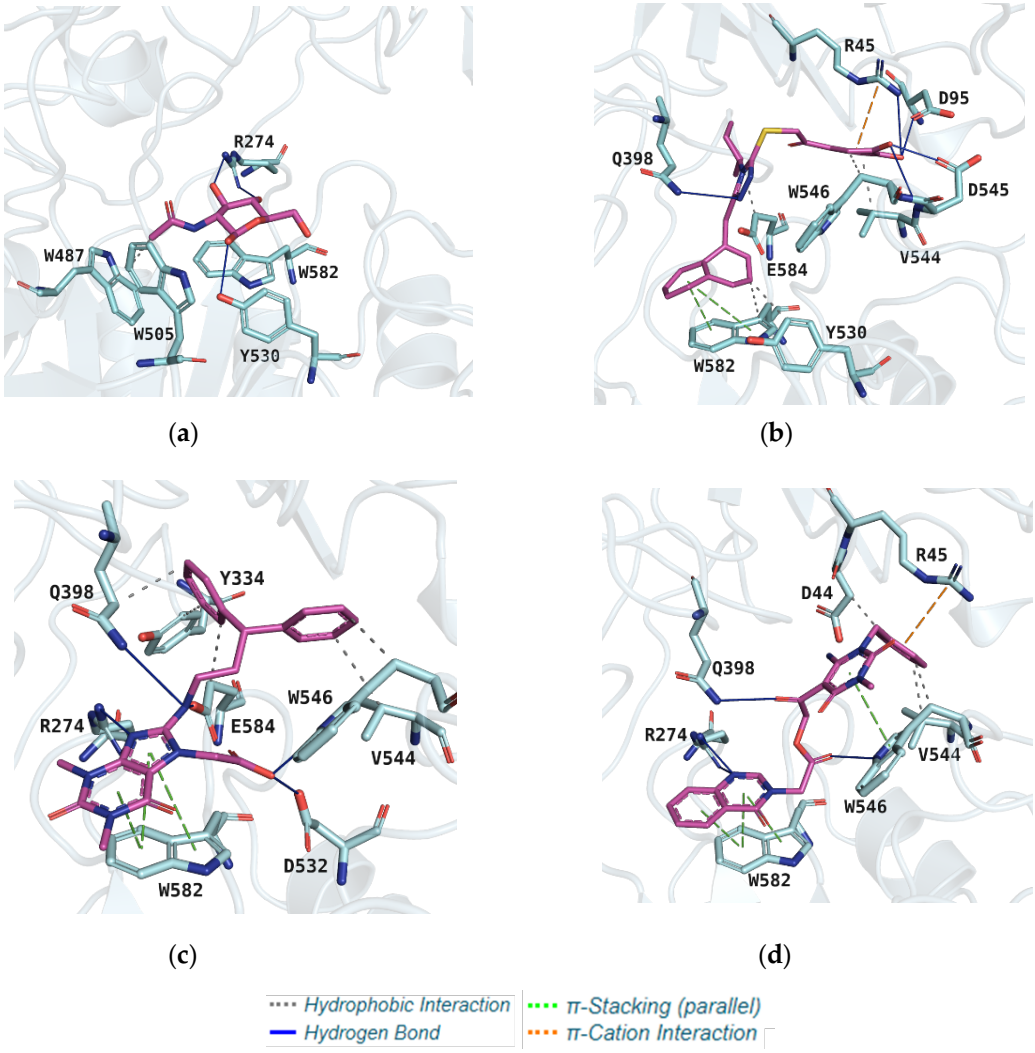


Figure 3. Interactions of (a) GlcNAc, (b) 338175, (c) 1146525 and (d) 340469 with the active-site residues of *Vh*GlcNAcase, obtained from the molecular docking study. GlcNAc, 338175, 1146525 and 340469 are shown as pink sticks, and active site residues of *Vh*GlcNAcase are shown as blue sticks.

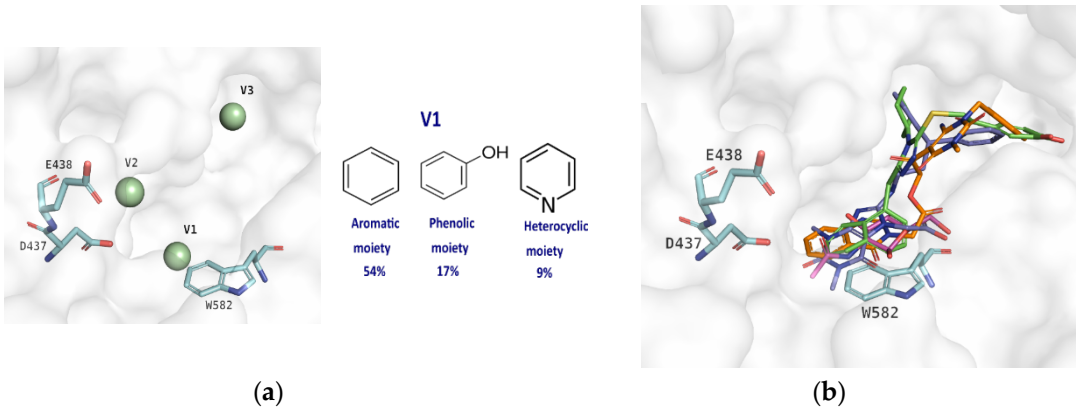


Figure 4. Pharmacophore analysis and superimposition of compounds with three highest ChemPLP scores. (**a**) Pharmacophore analysis by the V1, V2, and V3 anchor algorithm in SiMMap (**b**) Superimposition of crystal structure of GlcNAc (pink) and the docked structures of 338175 (green), 1146525 (blue), and 340469 (orange) in the *Vh*GlcNAcase (surface representation) binding site. The catalytic pair (D437/E438) and W582 that are located at subsite -1 are shown as blue sticks.

4. Discussion

In our virtual screening trials with more than 9,948 Reaxys compounds we successfully identified the ten compounds with the highest ChemPLP scores (98.27 to 104.15) in targeting GH-20 GlcNAcases. These compounds had ChemPLP scores higher than those of the reference compounds, $GlcNAc_4$ and Redoxal. Each of the top ten compounds can form intensive interactions with a strongly hydrophobic environment around subsite -1, including π-stacking and hydrogen bonds. All the identified compounds were found to form π-stacks with W582, which is conserved among the GlcNAcase family. π-stackings help to stabilize the interaction of cyclic rings of the bound ligand with the wall of the substrate-binding pocket, while hydrogen bonds generally help to stabilize interactions with charged residues in the pocket. Other interactions between the binding pocket and ligand, such as halogen bonds, salt bridges, hydrophobic and π-cation interactions, also occur. Of the previously reported potent inhibitors of *Vh*GlcNACase, such as Redoxal, PUGNAc and NAG-Thiazoline (NGT), all were found to fit well within site -1, the most critical subsite of the *Vh*GlcNACase-binding pocket. NGT occupies the -1 subsite by making contact through H-bonds with the sidechains of residues D437, Y530, H373, R274, D303, Glu584, D532 and W546. Importantly, W582 stacks directly against the plane of its pyranose ring [23]. PUGNAc also fits well within subsite −1 and forms hydrogen bonds with residues R274, Q398, D532 and E584, and hydrophobic interactions with W582 [8]. On the other hand, Redoxal is found to interact with three Trp residues, of which W487, W50 and W582 stack against the two benzene rings that occupy the inner part between subsites -1/-2 of the substrate-binding cleft and residues R274 and D303 form three hydrogen bonds with two oxygen atoms of the carboxyl group of the inner benzoic acid [13]. Clearly all three inhibitors form common interactions with W582, suggesting that this residue is essential for enzyme-drug interactions. Furthermore, we found that the inner ring of all top ten compounds identified in this study can form interactions with W582 within subsite -1 of *Vh*GlcNACase. Another common interaction is that R274 forms a hydrogen bond with the three known inhibitors and with the inner ring of 8 out of 10 identified compounds in this study (compounds 1146525, 340469, 251712, 1318389, 901602, 537731, 603310 and 527093). The results of virtual screening suggest that these ten compounds can serve as potential novel potent inhibitors of *Vh*GlcNACase. Pharmacophore analysis also shows, in agreement with interaction analysis, that the inner part of all the ten compounds is located at the bottom of the catalytic cavity of *Vh*GlcNAcase at position V1 while their outer parts stretch outwards from the binding pocket, with preferred interaction at position V3. However, it is well known that docking-based virtual screening is efficient in identifying hit compounds that have the potential to be potent inhibitors but not accurate in identifying the binding strength of each compound to the target protein. Thus, these ten hit compounds need to be studied further and determined for their efficiency and potency in suppressing GlcNAcases activity by further study.

5. Conclusions

In this study, we performed computational identification of novel GH-20 GlcNAcase inhibitors, using GOLD docking software. We report here ten potential inhibitors, all of which have a greater ChemPLP score than the known inhibitor Redoxal and the natural substrate $GlcNAc_4$. These compounds form two interactions in common with previously reported inhibitors, which also indicates the potential of these compounds to be novel inhibitors of GH-20 GlcNAcase. Interaction and pharmacophore analysis show that the inner part of these ten compounds make various interactions with the enzyme in the deep part of the binding pocket, while the outer part of the compounds stretch outward from the binding pocket and form various interactions near the surface of the enzyme, Thus, all ten compounds are candidates for further experimental study to verify their potential for further development as novel, highly potent GH-20 GlcNAcase inhibitors.

Author Contributions: G.P.; performed virtual screening, molecular docking of the Reaxys compound with *Vh*GlcNAcase, carried out pharmacophore analysis, data curation, and writing—original draft preparation. B.B; methodology, W.S.; validation, investigation, review, editing, supervision, and project administration. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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