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Computational Identification of Natural Product-Derived GSK-3 β Inhibitors as Candidate Therapeutics for Type 2 Diabetes and MASH

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approved the final draft and are responsible for the content and similarity index of the manuscript.

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ABSTRACT

Background: Type 2 Diabetes Mellitus (T2DM) and Metabolic Dysfunction-Associated Steatohepatitis (MASH) represent two converging metabolic epidemics that share interconnected pathophysiological mechanisms. Glycogen synthase kinase-3 beta (GSK-3 β) is a master metabolic regulator whose sustained hyperactivation drives both diseases, making it an attractive therapeutic target for both conditions.

Methods: This study employed a comprehensive in silico workflow to identify natural product-derived GSK-3 β inhibitors from the Natural Products Atlas. A total of 36,545 microbial natural products were initially screened for drug-likeness, followed by screening using an e-pharmacophore model derived from the GSK-3 β structure (PDB ID: 4ACC). The docking protocol was validated by re-docking and enrichment analysis. Lead compounds were further evaluated using MMGBSA binding free energy calculations, Induced Fit Docking (IFD), AutoQSAR, and ADMET predictions.

Results: The validated docking protocol exhibited exceptional discriminatory power (ROC = 0.98; AUC = 0.97; EF₅% = 100%). NPA011425, NPA014875, NPA020257, NPA004276, NPA034843, and NPA036064, with XP docking scores ranging from -6.858 to -11.059 kcal/mol, all superior to the co-crystallized ligand (-6.455 kcal/mol) and clinical inhibitors AR-A014418 (-5.929 kcal/mol) and Tideglusib (-5.895 kcal/mol). NPA014875 demonstrated the strongest binding free energy (-62.67 kcal/mol), comparable to the native ligand (-63.15 kcal/mol), AR-A014418 (-44.12 kcal/mol) and Tideglusib (-41.06 kcal/mol). Induced fit docking confirmed receptor-adaptive binding capabilities. All hits formed key hydrogen bonds with the conserved hinge-region residues (Asp133, Val135, and Pro136) and engaged the hydrophobic ATP-binding pocket. NPA020257 exhibited the highest predicted potency (pIC₅₀ = 6.82). ADMET profiling indicated that all six hits complied with Lipinski's rules and showed generally favorable pharmacokinetic profiles.

Conclusion: NPA020257, NPA034843, and NPA036064 emerged as the most balanced candidates due to favorable binding, pharmacokinetic profiles, and absence of major predicted toxicity liabilities. These findings highlight microbial natural products as promising scaffolds for GSK-3 β inhibitor development and support further molecular dynamics, enzymatic, and cellular validation.

INTRODUCTION

Type 2 Diabetes Mellitus (T2DM) and Metabolic Dysfunction-Associated Steatohepatitis (MASH), formerly designated Non-Alcoholic Steatohepatitis (NASH) have emerged as two of the most prevalent, clinically intertwined, and economically burdensome chronic metabolic disorders of the twenty-first century [1,2]. The International Diabetes Federation estimates that over 537 million adults worldwide are currently living with diabetes, with T2DM accounting for approximately 90–95% of all cases, and projections indicate this number will exceed 783 million by 2045 [3]. Concurrently, Metabolic Dysfunction-Associated Steatotic Liver Disease (MASLD) affects approximately 25–30% of the global adult population, and a significant subset of these individuals progress to MASH, which is characterized by hepatocellular steatosis, lobular inflammation, hepatocyte ballooning, and varying degrees of progressive perisinusoidal and portal fibrosis [4,5].

The clinical relationship between T2DM and MASH is profoundly reciprocal and pathophysiologically intertwined. Epidemiological data consistently demonstrate that more than 70% of patients with T2DM harbor concurrent MASLD, and approximately 30–40% of these individuals exhibit biopsy-confirmed MASH [6]. Conversely, the presence of MASH significantly accelerates the progression of hepatic and systemic insulin resistance, amplifies cardiovascular morbidity, and independently predicts the development and worsening of T2DM [7,8]. This bidirectional relationship creates a self-reinforcing pathogenic cycle in which each condition progressively exacerbates the other.

At the molecular level, insulin resistance serves as the central and unifying pathophysiological driver for both T2DM and MASH [9]. Under normal physiological conditions, insulin binding to the insulin receptor (IR) activates the insulin receptor substrate (IRS)/phosphoinositide 3-kinase

(PI3K)/protein kinase B (Akt) signaling cascade, which promotes glucose uptake in skeletal muscle and adipose tissue, stimulates hepatic glycogen synthesis, and suppresses hepatic gluconeogenesis and lipolysis [10]. However, in the setting of chronic caloric excess and obesity, dysfunctional and hypertrophied adipocytes undergo hyperlipolysis, releasing excessive free fatty acids (FFAs) into the systemic circulation. These FFAs accumulate ectopically in the liver and skeletal muscle, inducing lipotoxicity, endoplasmic reticulum (ER) stress, and mitochondrial dysfunction [11,12]. The resultant lipotoxic cellular environment impairs IRS phosphorylation at activating tyrosine residues while promoting inhibitory serine phosphorylation, thereby uncoupling the insulin receptor from its downstream effectors and perpetuating a vicious cycle of hyperglycemia, compensatory hyperinsulinemia, and accelerated hepatic de novo lipogenesis (DNL) [9,13].

Despite the profound clinical intersection of T2DM and MASH, current therapeutic options remain largely fragmented and disease-specific. While several classes of glucose-lowering agents are available for T2DM management, including metformin, sulfonylureas, dipeptidyl peptidase-4 (DPP-4) inhibitors, sodium-glucose cotransporter-2 (SGLT2) inhibitors, and glucagon-like peptide-1 (GLP-1) receptor agonists, many of these agents do not directly address the hepatic inflammatory and fibrotic cascades that drive MASH progression [14,15]. Conversely, the recently approved MASH therapeutic resmetirom (a thyroid hormone receptor- β agonist) primarily targets hepatic lipid metabolism and does not comprehensively correct systemic insulin resistance or peripheral glucose disposal [16]. This therapeutic fragmentation underscores the urgent clinical need for novel, mechanistically unified therapeutic agents capable of simultaneously targeting the shared molecular drivers of both conditions.

Glycogen Synthase Kinase-3 beta (GSK-3 β), a constitutively active serine/threonine protein kinase encoded by the GSK3B gene on chromosome 3q13.33, has emerged as one of the most compelling molecular targets at the pathophysiological intersection of T2DM and MASH [17, 18]. Originally identified and named for its role in inhibiting glycogen synthase (GS) through direct phosphorylation at serine residues 641/645, GSK-3 β is now recognized as a highly pleiotropic kinase that phosphorylates over 100 diverse substrates, regulating processes ranging from glucose and lipid metabolism to inflammation, cell survival, and fibrogenesis [19, 20]. Under physiological conditions, insulin receptor activation triggers the PI3K/Akt signaling

cascade, wherein Akt phosphorylates and inactivates GSK-3 β at Serine-9 (Ser9), thereby releasing the inhibitory phosphorylation on GS and enabling hepatic and skeletal muscle glycogen synthesis [21,22]. In the insulin-resistant states characterizing T2DM and MASH, however, attenuated PI3K/Akt signaling results in sustained, unrestrained GSK-3 β hyperactivation, which perpetuates metabolic dysfunction through multiple converging mechanisms [23].

In the context of glucose metabolism and T2DM, constitutively active GSK-3 β exerts multiple detrimental effects. First, it maintains glycogen synthase in its phosphorylated, inactive state, impairing glycogen synthesis and contributing to postprandial hyperglycemia [24,35]. Second, GSK-3 β directly phosphorylates IRS-1 at inhibitory serine residues, promoting its proteasomal degradation and attenuating upstream insulin signaling in a feed-forward manner that deepens systemic insulin resistance [26,27]. Third, GSK-3 β promotes hepatic gluconeogenesis through stabilization of the transcriptional co-activator PGC-1 α and the forkhead transcription factor FOXO1, driving the expression of rate-limiting gluconeogenic enzymes including phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) [28, 29]. Fourth, GSK-3 β phosphorylates and inactivates the glycogen-binding subunit of protein phosphatase 1 (PP1), further suppressing dephosphorylation and activation of glycogen synthase [30]. Collectively, these actions of GSK-3 β establish it as a central driver of the hyperglycemia and hepatic glucose overproduction that characterize T2DM. Accordingly, preclinical genetic and pharmacological studies consistently demonstrate that selective GSK-3 β inhibition or knockout results in markedly improved insulin sensitivity, enhanced glucose disposal, increased glycogen synthesis, and suppressed hepatic glucose output in rodent models of T2DM [31,32].

In the context of hepatic lipid metabolism and MASH, GSK-3 β contributes to disease progression through multiple pathways. It has been shown to modulate SREBP-1c-mediated lipogenesis, impair AMPK-driven fatty acid oxidation, and redirect glucose-derived carbon flux toward hepatic triglyceride synthesis [33,34]. Beyond steatosis, GSK-3 β plays a central role in the inflammatory progression that distinguishes MASH from simple steatosis. Through phosphorylation and degradation of I κ B, GSK-3 β sustains NF- κ B activation, amplifying the transcription of pro-inflammatory cytokines including TNF- α , IL-6, and IL-1 β in hepatocytes and Kupffer cells [35,36]. In hepatic stellate cells, GSK-3 β promotes TGF- β /Smad-driven

fibrogenic gene expression and suppresses anti-fibrotic Wnt/ β -catenin signaling, thereby accelerating collagen deposition and hepatic fibrosis [37, 38]. Preclinical evidence from multiple rodent models of MASLD/MASH demonstrates that pharmacological inhibition or genetic deletion of GSK-3 β attenuates hepatic steatosis, reduces inflammatory cytokine expression, and limits fibrotic progression [39, 40].

Taken together, the central role of GSK-3 β in governing the shared molecular pathophysiology of T2DM and MASH positions it as an exceptionally attractive target for dual-action therapeutic intervention. Despite this promise, the clinical translation of GSK-3 β inhibitors has been impeded by several challenges, including insufficient isoform selectivity between GSK-3 α and GSK-3 β , potential oncogenic risks from Wnt/ β -catenin pathway hyperactivation, and systemic toxicities associated with first-generation synthetic inhibitors [41, 42]. These limitations underscore the urgent need for novel, selective, and structurally diverse GSK-3 β inhibitors with favorable safety profiles.

Throughout the history of pharmacotherapy, natural products have served as an unparalleled source of chemical diversity and biological activity for drug discovery [43]. Unlike synthetic compound libraries that are often constrained to narrow, "drug-like" chemical space defined by combinatorial chemistry approaches, natural products occupy privileged regions of chemical space characterized by exceptional stereochemical complexity, high fraction of sp³-hybridized carbons, diverse ring systems, and a balanced distribution of hydrophilic and hydrophobic functional groups [44,45]. These structural attributes confer superior target complementarity and biological membrane permeability, translating into favorable drug-likeness and reduced toxicity profiles [46]. The pharmaceutical significance of natural products is underscored by the observation that approximately 50% of all small-molecule drugs approved by the United States Food and Drug Administration (US FDA) between 1981 and 2019 are either unmodified natural products, semi-synthetic natural product derivatives, or synthetic compounds designed around natural product pharmacophores [43].

The Natural Products Atlas (NP Atlas) is an open-access, comprehensively curated database containing structural, taxonomic, and biosynthetic data for over 36,000 small molecules of verified microbial origin, encompassing both bacterial and fungal secondary metabolites [47]. The NP Atlas provides a strategically optimized chemical library for computational drug

discovery, enabling the systematic virtual screening of structurally diverse and evolutionarily refined microbial metabolites against therapeutic targets of interest. Exploring this repository computationally offers an efficient, cost-effective, and non-destructive strategy to identify structurally novel GSK-3 β inhibitor scaffolds that bypass the chemical redundancy often encountered in traditional synthetic screening libraries. By employing a comprehensive in silico pipeline, this study aims to identify novel, potent, and structurally diverse microbial-derived GSK-3 β inhibitors from the Natural Products Atlas database. These identified molecules will serve as promising candidate therapeutics for the concurrent management of Type 2 Diabetes and MASH.

MATERIALS AND METHODS

Target library

Bioactive natural compounds were obtained from the NATURAL Products Atlas (<https://www.npatlas.org/>), an open access database containing information on small molecules of microbial origin. The target library data set contains 36, 545 compounds that were retrieved in Structure Data File (SDF) format.

Drug-likeness analysis

The drug-likeness predictions of the target library compounds was done by applying Lipinski rule of five (RO5) parameters ($MW \leq 500$, $LogP \leq 5$, $HBD \leq 5$, $HBA \leq 10$) [48]. In this study, the drug-likeness properties of the compounds were calculated using DataWarrior v06.05.04. A total of 21, 390 compounds passed the screening.

E-pharmacophore generation and Screening

A pharmacophore model was constructed using the receptor–ligand complex structure of co-crystallized ligand and human glycogen synthase kinase-3 beta (GSK-3 β). The model has four unique features. The resulting 21, 390 compounds were filtered using strict screening criteria (4 out of 4 features) in an effort to find potential lead compounds with the right pharmacophoric properties. A total of 218 compounds passed the screening.

Ligand preparation

The resulting 218 compounds that fit with the expected pharmacophore theory were prepared using the LigPrep module of Schrödinger Maestro 12.8. The ligands were processed by creating ionization states by adding or removing H⁺ to neutralize functional group charge where possible using Epik. Stereoisomers were generated while retaining specified chiral centers where applicable, followed by geometry optimization using the OPLS4 force field. AR-A014418 and Tideglusib were selected as reference inhibitors.

Protein preparation

The crystal structure of glycogen synthase kinase-3 beta (GSK-3 β) [PDB ID: 4ACC] was retrieved from Protein Data Bank (<http://rcsb.org>). The protein preparation was carried out with Protein Preparation Wizard in Maestro Schrödinger (pH=7.0 $\pm$ 2.0), that includes bond orders assignment, addition of hydrogen atoms, side chain correction, hydrogen bonding network optimization and removal of crystallographic water molecules outside the 5 Å range or more from the active site. The protein structure was energetically minimized with the OPLS4 force field in order to achieve a stable conformation for molecular docking studies.

Grid generation

The active site of the target protein was determined based on the position of native ligand that was present within the glycogen synthase kinase-3 beta binding pocket using the receptor grid generation module of Maestro Schrödinger Suite. The scoring coordinates; x, y, z grid are 17.46, 18.86, and 10.74 respectively.

Validation of docking protocol

The docking protocol was validated through re-docking (superimposition) and enrichment analysis. First, the methodology was validated by calculating the root mean square deviation (RMSD) from the native ligand, extracted from the glycogen synthase kinase-3 beta complex and re-docked into the binding site. To validate the virtual screening workflow, 25 known inhibitors of glycogen synthase kinase-3 beta were retrieved from the ChEMBL database

(<https://www.ebi.ac.uk/chembl/>). The curated actives were formatted as SMILES strings and submitted to LUDe (LIDEB's Useful Decoys <https://lideb-lude-v2.streamlit.app/#fast-tutorial-lude>) to generate 1,250 putative inactive compounds (decoys). The LUDe WebApp was run using default configurations. The resulting decoy set was retrieved in "CSV" format and transformed into "SDF" format using DataWarrior software. The actives and decoys were prepared using the Ligand Preparation panel in the Maestro Schrödinger workspace, followed by receptor-ligand docking. The final validation set, comprising 1,275 compounds (1,250 decoys and 25 active ligands), was used to evaluate the model's integrity. The enrichment report analyzed key parameters, including overall performance (ROC and AUC), BEDROC value, Enrichment Factor, Robust Initial Enhancement, Recovery rate and ROC curve. While the EF measures the recovery of true positives relative to the decoys, the ROC evaluates the trade-off between true positives (sensitivity) and false positives (1-specificity).

Structure-based screening

The generated 218 compounds that passed the pharmacophore screening were subjected to further screening utilizing molecular docking techniques with varying degrees of precision. The list was first reduced to 178 compounds using HTVS (High Throughput Virtual Screening), and then 88 compounds were filtered using a docking threshold of -6.0 kcal/mol. The list was then reduced to 69 compounds by using Standard Precision (SP) docking with a more stringent threshold score of -6.5 kcal/mol. Finally, the top 50 compounds from SP docking were subjected to XP docking, from which six top-ranked compounds were selected for further analysis.

MMGBSA calculations

Molecular docking was further validated using MMGBSA calculations developed by Kollman et al. [49], which offer a more reliable alternative for estimating free binding energy. The binding free energy of the docked complexes was determined using the Molecular Mechanics Generalized Born Surface Area (MMGBSA) tool coupled with prime of the Maestro Schrödinger. The OPLS4 force field and VSGB solvent were used to calculate the relative free energy of the docked complexes. This analysis aimed to estimate the relative binding free energies of the ligands toward the selected protein.

Induced fit docking (IFD) - Extra Precision

To account for binding site flexibility, selected ligands were subjected to XP-IFD, generating poses using a 0.50 van der Waals (vdW) scaling factor for both receptor and ligand, and calculations followed the standard protocol in Schrödinger.

Auto-QSAR

This study employed the Schrödinger AutoQSAR framework, which automatically optimizes hyperparameters with minimal user input. The framework generates numerical or categorical models using a combination of topological descriptors, physicochemical properties, and various 2D molecular fingerprints (radial, linear, and dendritic) [50]. Experimentally reported GSK-3 β [PDB ID: 4ACC] inhibitors were retrieved from the ChEMBL database (<https://www.ebi.ac.uk/chembl/>) using the protein's FASTA sequence and curated for QSAR model generation. Subsequently, the resulting list of selected inhibitors (1,500 compounds) was converted to SDF format using DataWarrior software [51]. The SDF file was imported into the workspace of Maestro Schrödinger and prepared using the ligand preparation module. These prepared ligands were then used to build QSAR model using the AutoQSAR module. Among the generated models, the best model was determined based on ranking, leading to the selection of the kpls_radial_32 model. This chosen model was subsequently applied to predict the pIC₅₀ of the hit compounds highlighted in the research.

ADMET prediction

The pharmacokinetics properties of the top compounds were evaluated using the SwissADME online server database (<https://www.swissadme.ch/>) and the ProTox-3.0 web server (<https://tox.charite.de/protox3/>) for toxicity prediction. The study was carried out to evaluate the pharmacokinetic profile of the docked compounds, including physiochemical properties, drug-likeness, medicinal properties, and toxicity profile, which are essential for assessing the potential effectiveness and safety of drug candidates.

RESULTS

Drug-likeness Analysis

From the initial Natural Products Atlas library comprising 36,545 bioactive natural compounds, a total of 21,390 compounds (58.5%) satisfied Lipinski's Rule of Five (RO5) criteria as implemented in DataWarrior. These RO5-compliant compounds constituted the filtered target library that was subsequently advanced to pharmacophore screening.

E-Pharmacophore Generation and Screening

An e-pharmacophore model was constructed from the receptor–ligand complex of human glycogen synthase kinase-3 beta (GSK-3 β ; PDB ID: 4ACC) and its co-crystallized ligand. The resulting model comprised four unique features: three aromatic ring and one hydrogen bond donor, with respective energy contributions of -0.66 , -0.89 , -1.02 , and -0.70 kcal/mol (Figure 1). Screening of the 21,390 drug-like compounds against this four-feature pharmacophore using strict matching criteria (4 out of 4 features) yielded 218 compounds (1.0% of the filtered library) that possessed the requisite spatial and electronic properties for potential GSK-3 β inhibition. Following this screening, compounds that successfully matched all required pharmacophoric features and were selected for structure-based virtual screening.

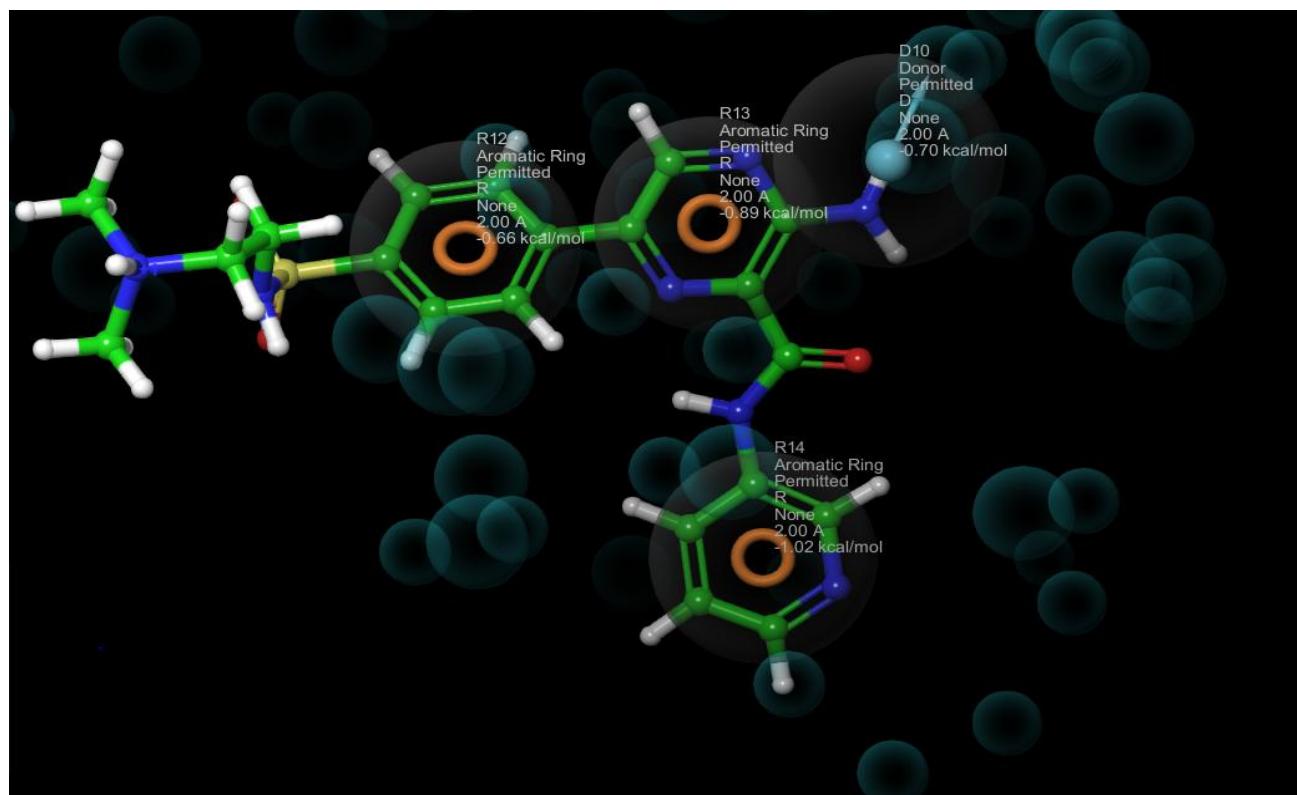


Figure 1: A pharmacophore model used to screen for compounds with potential inhibitory effect against glycogen synthase kinase-3 beta (GSK-3 β) [PDB ID: 4ACC].

Validation of the Molecular Docking Protocol

To ensure the reliability and predictive power of the docking workflow, the protocol was validated using both re-docking (pose reproduction) and enrichment analysis. First, the co-crystallized ligand was extracted from the GSK-3 β binding pocket and re-docked into the active site. The superimposition of the re-docked pose onto the native crystallographic pose yielded a Root Mean Square Deviation (RMSD) of 2.2831 Å (Figure 2). While slightly above the conventional 2.0 Å threshold, the key hinge-binding interactions were retained despite an RMSD slightly above 2.0 Å.

Second, an enrichment analysis was performed to evaluate the ability of the docking protocol to distinguish known active inhibitors from inactive decoys. Several statistical metrics were calculated to assess the docking procedure, as detailed in Supplementary Table 1. The Receiver Operating Characteristic (ROC) value, which ranges 0 to 1, was found to be 0.98, and was further supported by the ROC plot shown in Figure 3. The Area Under the Curve (AUC) value

of 0.97 (97%), indicates exceptional discriminatory power between actives and inactives. Furthermore, a total of 25 active ligands were included, and the enrichment factor at 5% indicated that all active compounds were recovered within the top 5% of ranked results. The RIE value was determined to be 12.27, and the BEDROC values at various tuning parameters were ($\alpha = 160.9/0.341$), ($\alpha = 20.0/0.741$), and ($\alpha = 8.0/0.883$). The minimum Tanimoto coefficient (T_c) among active ligands was 0.0093, indicating high structural diversity within the active set used for enrichment validation.

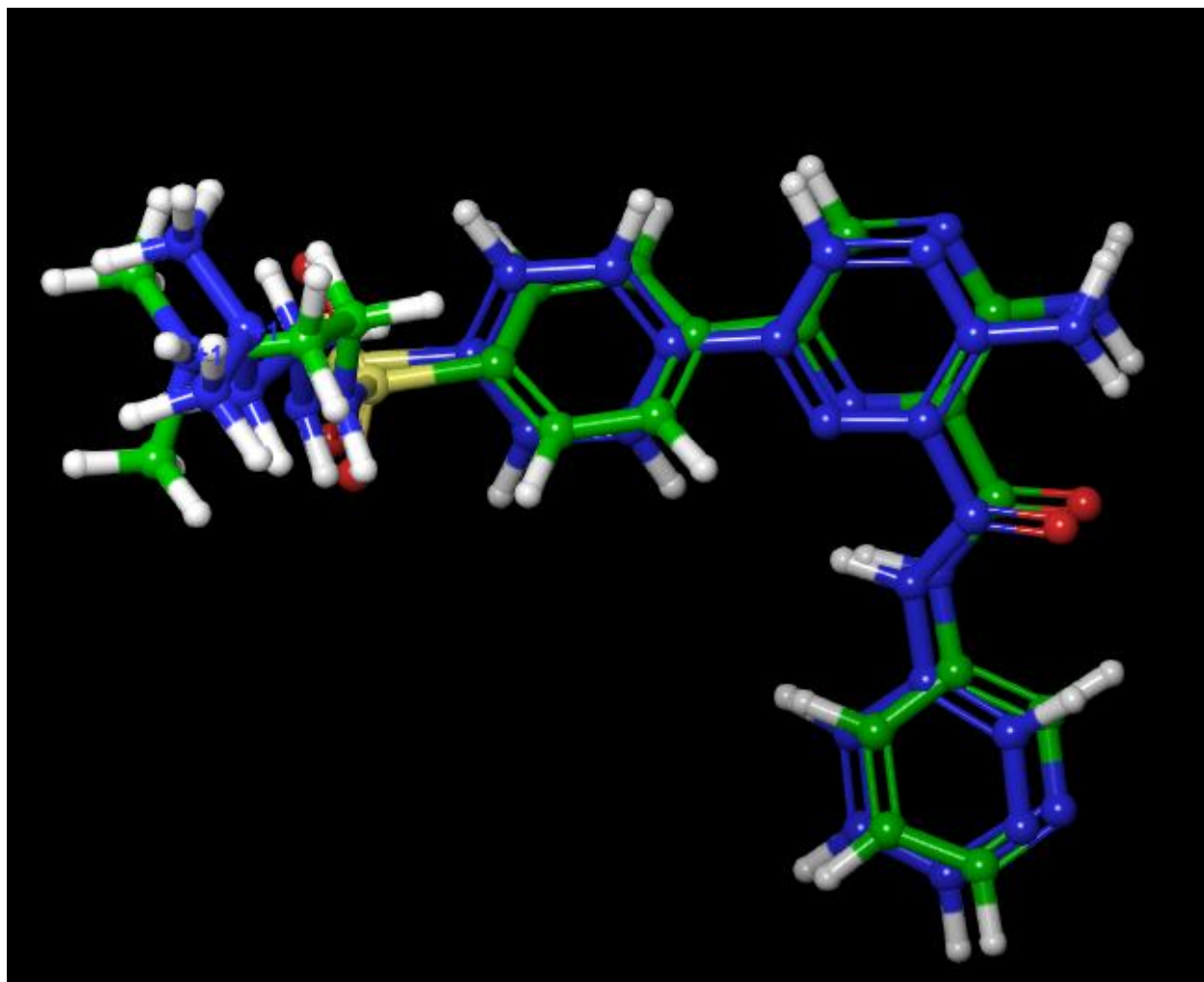


Figure 2: Superimposition of co-crystallized (green) and docking (blue) poses of the same ligands (RMSD = 2.2831 Å).

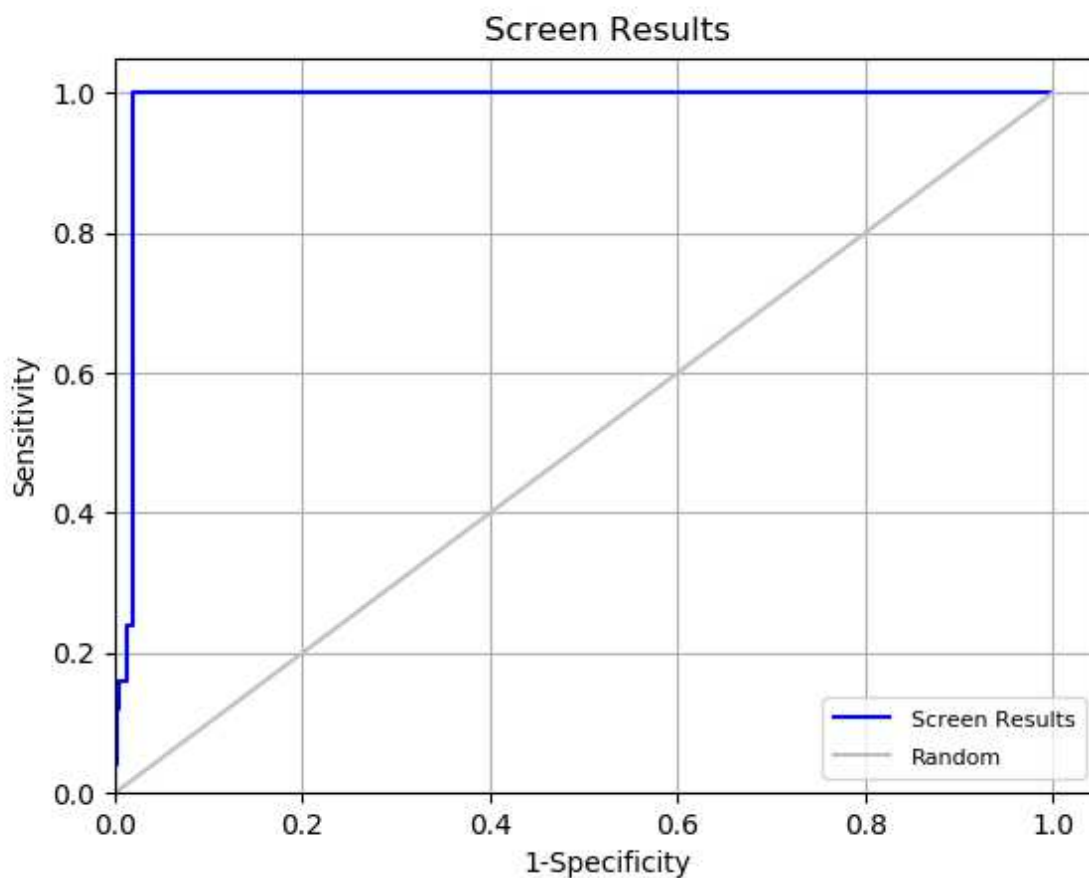


Figure 3: Receiver operating characteristics curve (ROC) plot (Enrichment analysis)

Structure-based Virtual Screening and XP Docking

The XP docking scores and pharmacophore fitness scores are summarized in Table 1. NPA011425 exhibited the most favorable XP docking score of -11.059 kcal/mol (fitness score: 2.105), markedly surpassing the co-crystallized reference ligand 7YG_4ACC_REF (-6.455 kcal/mol), AR-A014418 (-5.929 kcal/mol), and the clinical candidate Tideglusib (-5.895 kcal/mol). NPA014875 ranked second with a docking score of -9.545 kcal/mol (fitness: 1.498), followed by NPA020257 (-8.221 kcal/mol; fitness: 1.591), NPA004276 (-7.854 kcal/mol; fitness: 1.367), NPA034843 (-7.166 kcal/mol; fitness: 1.387), and NPA036064 (-6.858 kcal/mol; fitness: 1.720). All six natural product hits outperformed the reference ligand and both comparator inhibitors in terms of XP docking score. Notably, the co-crystallized reference ligand

displayed the highest fitness score (2.376), indicating optimal pharmacophore feature alignment despite its lower predicted binding score.

The 2D interaction diagrams of the XP-docked complexes (Figure 4) and the corresponding residue-level interaction summary (Table 2) revealed that all hit compounds engaged conserved residues within the GSK-3 β ATP-binding pocket. Common interaction partners included hinge region residues (ASP133, VAL135, PRO136), hydrophobic pocket residues (ILE62, VAL70, ALA83, LEU132, TYR134, LEU188, VAL110, CYS199, PHE67), and charged residues (GLU137, ASP200, ARG141, LYS85).

Interestingly, all six hits engage the conserved hinge region (VAL135/PRO136) similar to reference inhibitors, supporting ATP-competitive binding mode. NPA011425 formed hydrogen bonds with PRO136, VAL135, and a bidentate interaction with ASP133 (two H-bonds), while engaging in extensive hydrophobic contacts with eleven residues. NPA014875 established four hydrogen bonds, the highest among the hits with ARG141, PRO136, VAL135, and ASP133, and similarly engaged an extensive hydrophobic network. NPA020257 exhibited H-bonds with ASP200, GLN185, and ILE62; the engagement of GLN185 is notable as this residue lies within the substrate-recognition groove. NPA004276 was distinguished by a unique π - π stacking interaction with PHE67, alongside H-bonds with ASP200, ASP133, and SER66. NPA034843 formed H-bonds with PRO136, ARG141, and ILE62, the latter representing P-loop engagement. NPA036064 engaged PRO136 and ARG141 through hydrogen bonding and displayed the broadest polar residue network, involving GLN185, ASN186, ASN64, and THR138.

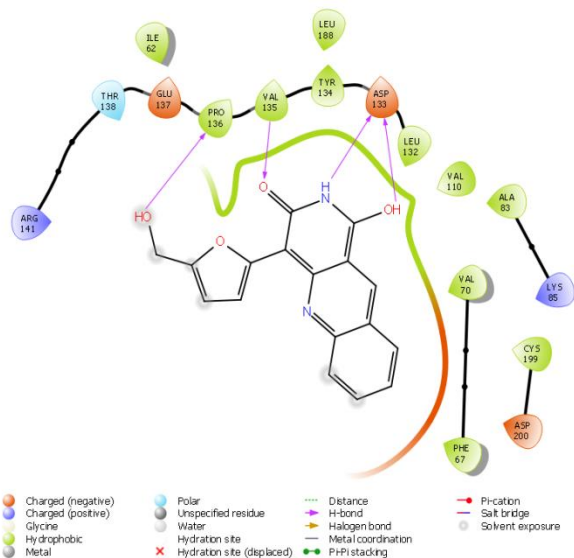
In comparison, the co-crystallized reference ligand formed H-bonds with ASP133, ARG141, and LYS85. AR-A014418 engaged PRO136 and VAL135 (bidentate), while Tideglusib formed a single H-bond with LYS85, consistent with its non-ATP-competitive binding mechanism.

Table 1: Docking and Fitness Scores of the Lead Compounds

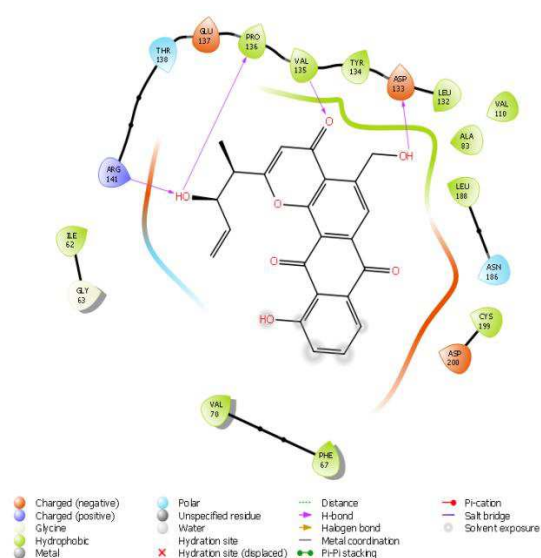
NPA ID/Entry name	Docking score (kcal/mol)	Fitness score
NPA011425	-11.059	2.105

NPA014875	-9.545	1.498
NPA020257	-8.221	1.591
NPA004276	-7.854	1.367
NPA034843	-7.166	1.387
NPA036064	-6.858	1.72
7YG_4ACC_REF (Reference)	-6.455	2.376
AR-A014418	-5.929	
Tideglusib	-5.895	

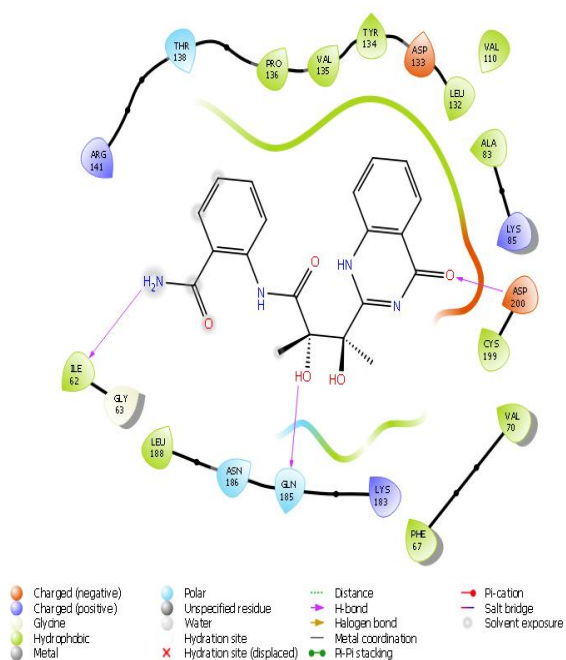
NPA011425



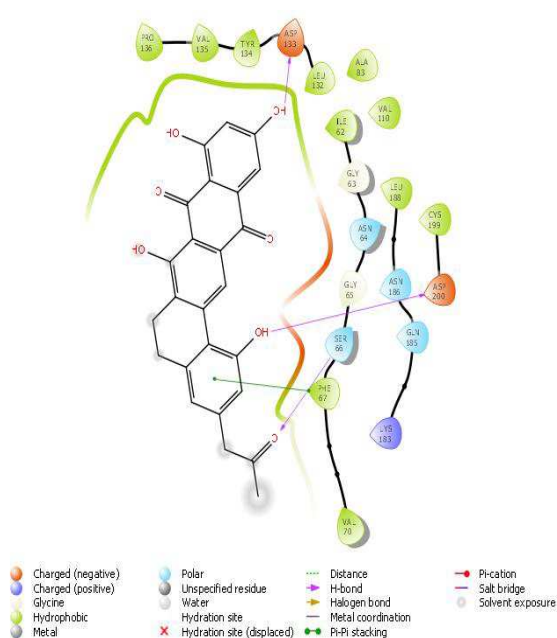
NPA014875



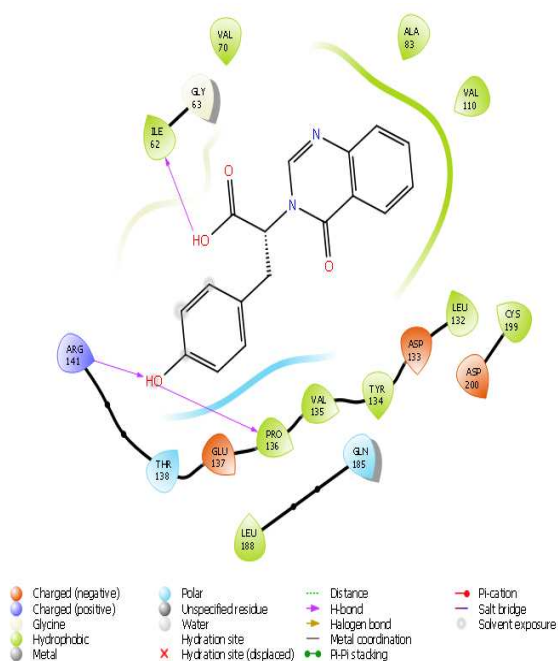
NPA020257



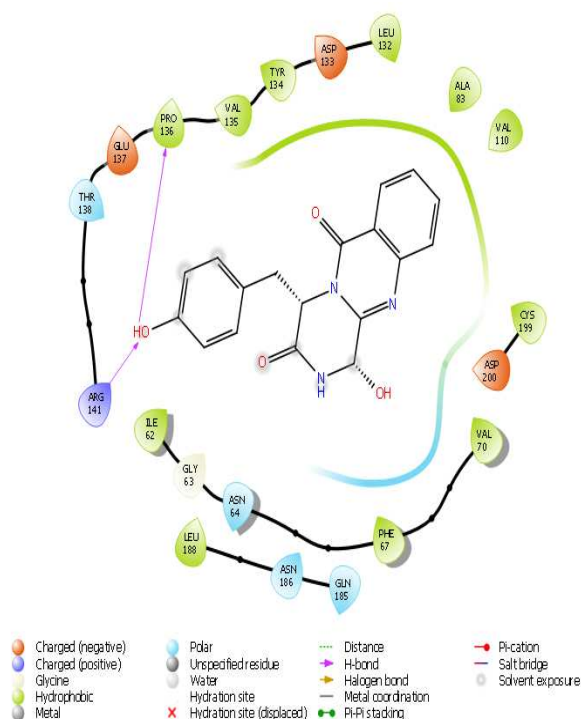
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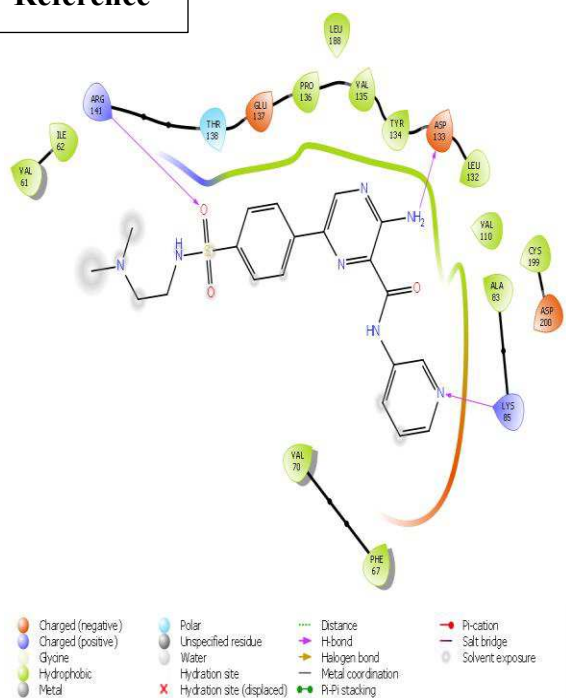
NPA034843



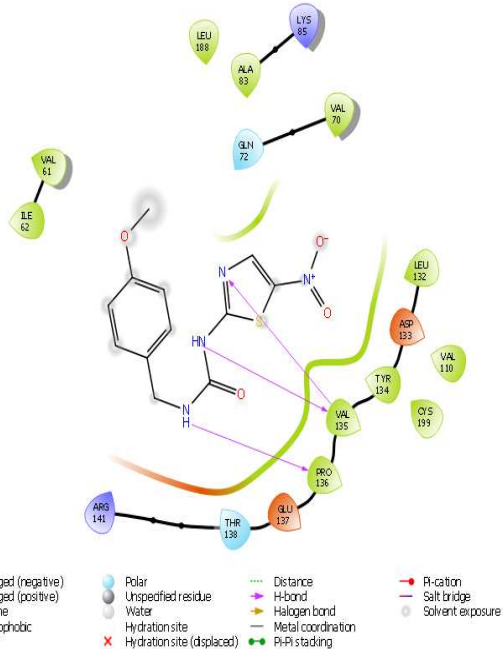
NPA036064



Reference



AR-A014418



Tideglusib

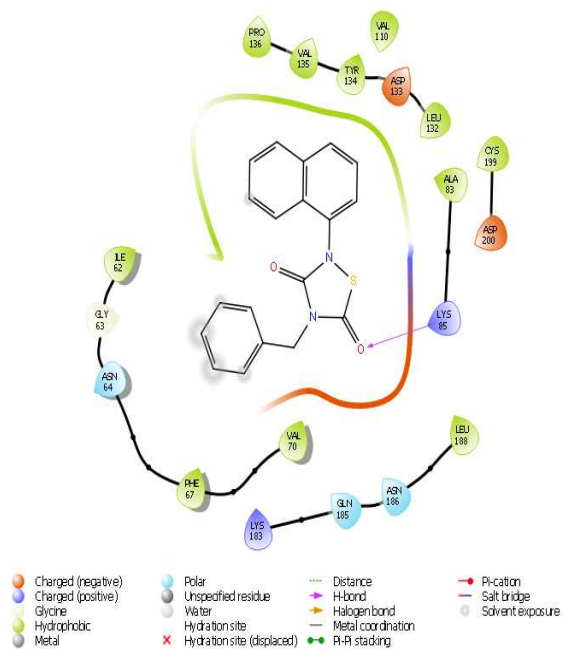


Figure 4: The 2D interaction of the protein-ligand complexes (XP Docking)

Table 2: Interaction of ligands with protein

NPA ID/Entry name	H-Bond Residues	Residues	Other Interactions
NPA011425	PRO136, VAL135, ASP133 (2).	Charged negative: GLU137, ASP133, ASP200. Charged positive: ARG141, LYS85. Polar: THR138 Hydrophobic/Aromatic: ILE62, PRO136, VAL135, TYR134, LEU188, LEU132, VAL110, ALA83, VAL70, CYS199, PHE67,	NONE
NPA014875	ARG141, PRO136, VAL135, ASP133.	Charged negative: GLU137, ASP133, ASP200. Charged positive: ARG141. Polar: THR138, ASN186. Hydrophobic/Aromatic: ILE62, PRO136, VAL135, TYR134, LEU132, VAL110, ALA83, LEU188, CYS199, PHE67, VAL70.	NONE
NPA020257	ASP200, GLN185, ILE62.	Charged negative: ASP133, ASP200. Charged positive: ARG141, LYS85, LYS183. Polar: GLN185, ASN186, THR138.	NONE

		Hydrophobic/Aromatic: LEU188, ILE62, PRO136, VAL135, TYR134, LEU132, VAL110, ALA83, CYS199, VAL70, PHE67.	
NPA004276	ASP200, ASP133, SER66.	Charged negative: ASP133, ASP200. Charged positive: LYS183. Polar: ASN64, ASN186, GLN185, SER66. Hydrophobic/Aromatic: PRO136, VAL135, TYR134, LEU132, ALA83, ILE62, VAL110, LEU188, CYS199, PHE67, VAL70.	Pi-Pi stacking (PHE67)

NPA034843	PRO136, ARG141, ILE62.	Charged negative: GLU137, ASP133, ASP200. Charged positive: ARG141, Polar: THR138, GLN185. Hydrophobic/Aromatic: ILE62, VAL70, ALA83, VAL110, LEU132, CYS199, TYR134, VAL135, PRO136, LEU188.	NONE
NPA036064	PRO136, ARG141.	Charged negative: GLU137, ASP133, ASP200. Charged positive: ARG141. Polar: GLN185, ASN186, ASN64, THR138. Hydrophobic/Aromatic: PRO136, VAL135, TYR134, LEU132, ALA83, VAL110, CYS199, VAL70, PHE67, LEU188, ILE62.	NONE

7YG_4ACC_REF (Reference)	ASP133, ARG141, LYS85.	Charged negative: GLU137, ASP133, ASP200. Charged positive: ARG141, LYS85. Polar: THR138. Hydrophobic/Aromatic: VAL61, ILE62, PRO136, VAL135, TYR134, LEU132, VAL110, CYS199, ALA83, PHE67, VAL70.	NONE
AR-A014418	PRO136, VAL135 (2).	Charged negative: GLU137, ASP133. Charged positive: ARG141, LYS85. Polar: GLN72, THR138. Hydrophobic/Aromatic: ILE62, VAL61, LEU188, ALA83, VAL70, LEU132, VAL110, TYR134, CYS199, VAL135, PRO136.	NONE
Tideglusib	LYS85	Charged negative: ASP133, ASP200. Charged positive: LYS85. Polar: ASN64, GLN185, ASN186. Hydrophobic/Aromatic: PRO136, VAL135, TYR134, VAL110, LEU132, ALA83, CYS199, LEU188, VAL70, PHE67, ILE62.	NONE

MMGBSA Binding Free Energy Calculations

The binding free energies of the docked complexes were further evaluated using MMGBSA calculations. The calculated binding free energies are presented in Table 3. The co-crystallized reference ligand exhibited the most favorable ΔG_{bind} of -63.15 kcal/mol. Among the natural product hits, NPA014875 achieved a ΔG_{bind} of -62.67 kcal/mol, essentially equivalent to the native ligand and substantially superior to all other hits and reference inhibitors. NPA004276 (-52.49 kcal/mol) and NPA020257 (-52.27 kcal/mol) demonstrated the next most favorable binding free energies, followed by NPA011425 (-49.11 kcal/mol), NPA034843 (-43.43 kcal/mol), AR-A014418 (-44.12 kcal/mol), Tideglusib (-41.06 kcal/mol), and NPA036064 (-40.16 kcal/mol). All natural product hits except NPA036064 exhibited MMGBSA energies superior to Tideglusib, and five of the six surpassed AR-A014418.

Table 3: MMGBSA Calculation of the docked complexes

NPA ID/Entry name	MMGBSA dG Bind (kcal/mol)
NPA011425	-49.11
NPA014875	-62.67
NPA020257	-52.27
NPA004276	-52.49
NPA034843	-43.43
NPA036064	-40.16
7YG_4ACC_REF (Reference)	-63.15
AR-A014418	-44.12

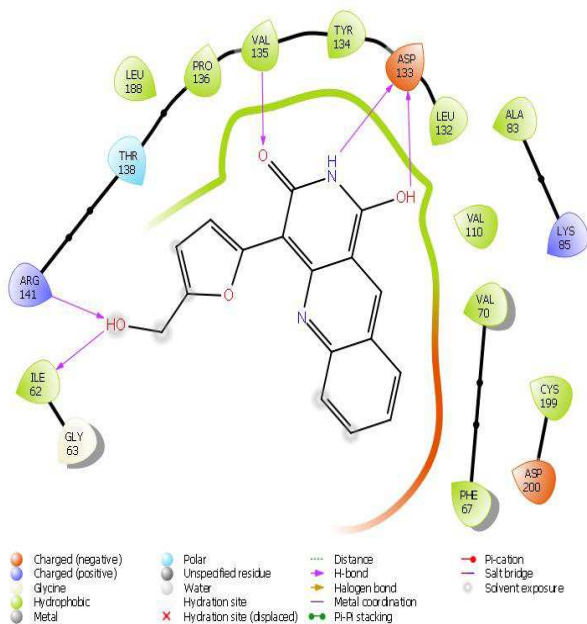
Tideglusib	-41.06
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Induced Fit Docking and Comparative Interaction Analysis

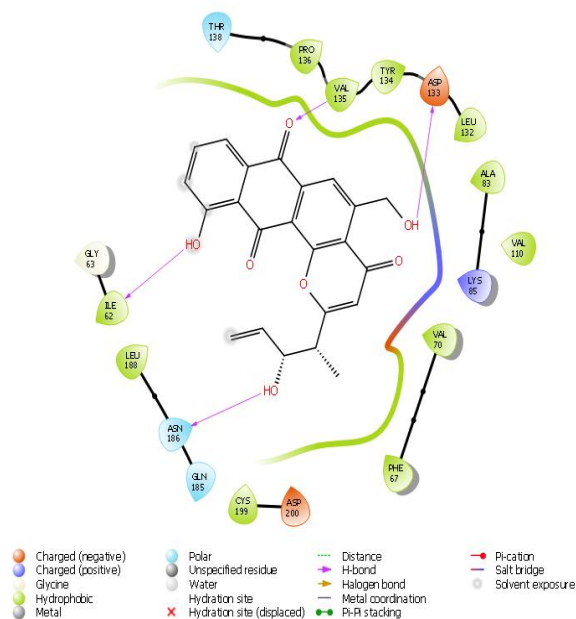
To account for receptor flexibility, the six hit compounds and reference ligands were subjected to Induced Fit Docking (IFD) with Extra Precision (XP) scoring (Table 4; Figure 5). IFD scores ranged from -731.11 kcal/mol (AR-A014418) to -722.68 kcal/mol (Tideglusib). NPA014875 achieved an IFD score of -730.74 kcal/mol, comparable to AR-A014418 and superior to the co-crystallized reference (-728.74 kcal/mol) and Tideglusib (-722.68 kcal/mol). NPA020257 (-728.92 kcal/mol) also performed comparably to the reference ligand under these docking conditions.

A detailed comparison of XP versus IFD interaction patterns (Table 5) revealed both conserved and adaptive binding features. Several compounds gained functionally significant interactions upon receptor relaxation. For instance, NPA011425 acquired additional H-bonds with ARG141 and ILE62 in IFD, while NPA004276 shifted its π - π stacking from PHE67 (XP) to TYR134 (IFD) and gained cationic contacts with ARG141 and LYS85. NPA034843 gained π - π stacking with PHE67 under IFD conditions, and NPA036064 acquired a π -cation interaction with ARG141. Conversely, some polar interactions observed in rigid docking (such as THR138 engagement in the reference ligand) were lost upon induced fit, reflecting receptor conformational rearrangement.

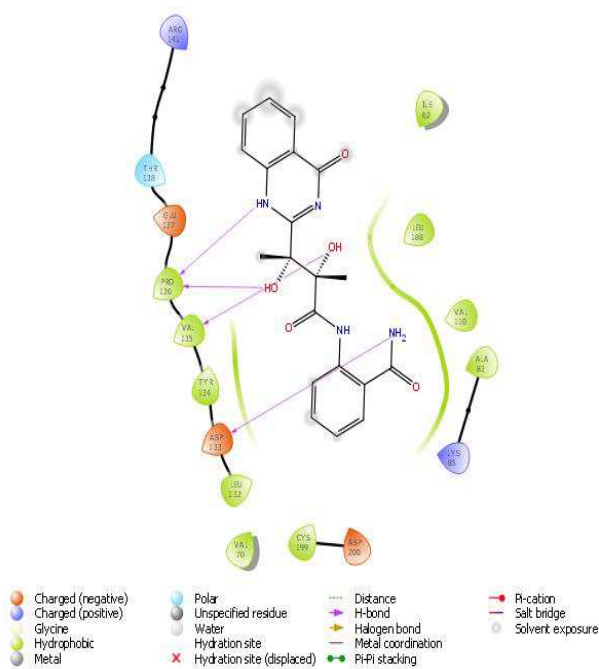
NPA011425



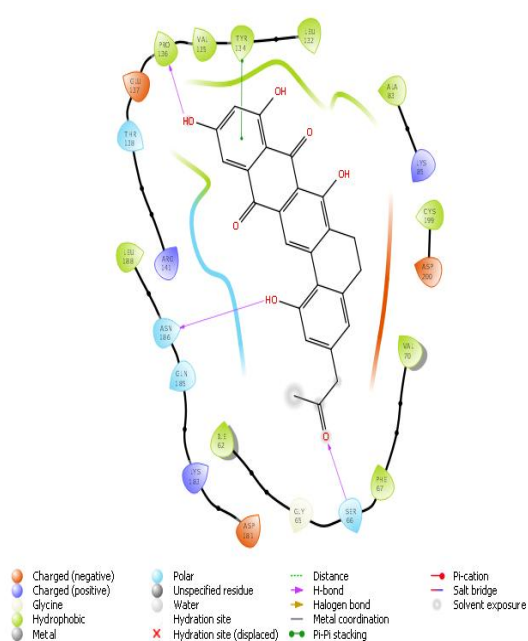
NPA014875



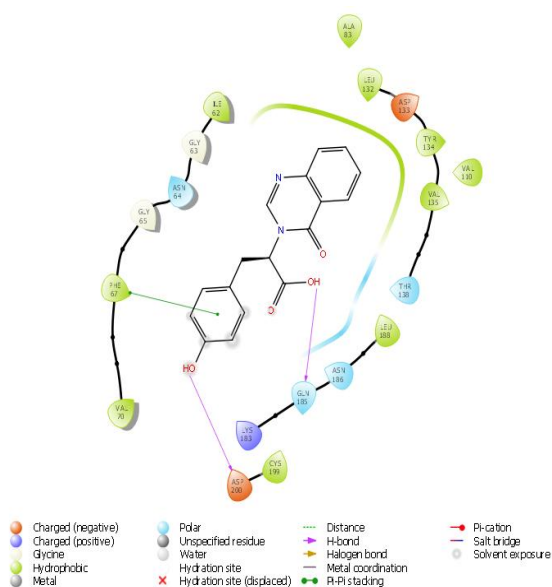
NPA020257



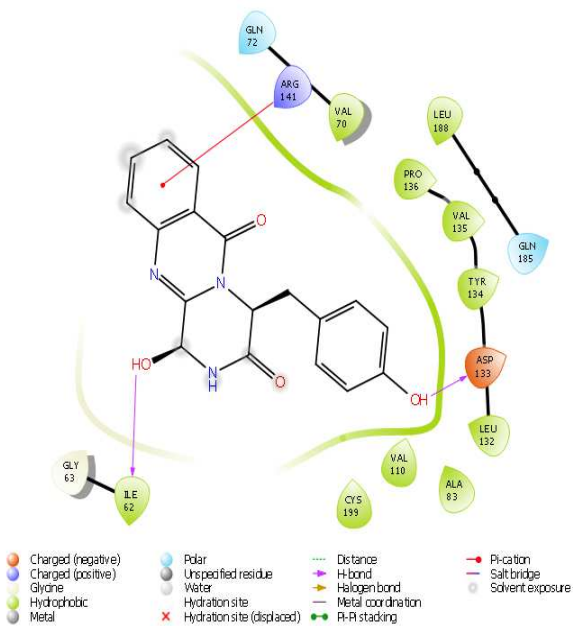
NPA004276



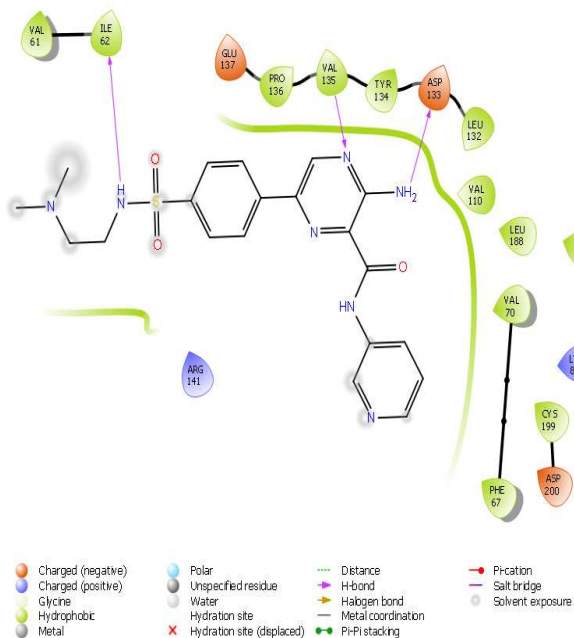
NPA034843



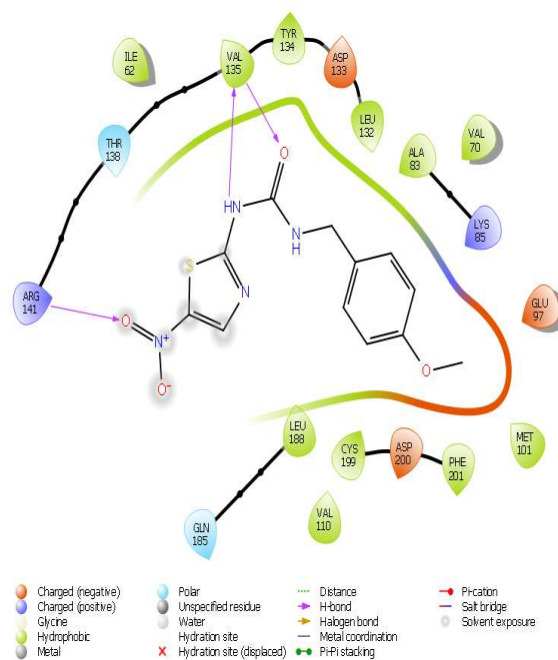
NPA036064



Reference



AR-A014418



Tideglusib

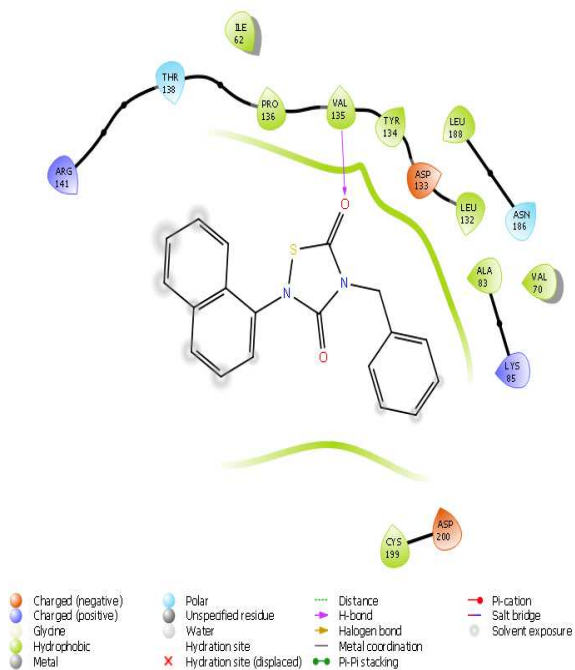


Figure 5: 2D interaction diagrams of the protein–ligand complexes (IFD-XP Docking)

Table 4: Induced Fit Docking

NPA ID/Entry name	IFD Score (kcal/mol)
NPA011425	-728.14
NPA014875	-730.74
NPA020257	-728.92
NPA004276	-724.4
NPA034843	-726.55
NPA036064	-726.66
7YG_4ACC_REF (Reference)	-728.74
AR-A014418	-731.11
Tideglusib	-722.68

Table 5: Summary of difference between interaction of ligands with protein complex in XP docking and induced fit docking (XP)

NPA ID/Entry name	Interaction shown in XP docking	Interaction shown in induced fit docking
NPA011425	Charged negative: GLU137, ASP133, ASP200. Charged positive: ARG141, LYS85. Polar: THR138 H-bond: PRO136, VAL135, ASP133 (2). Hydrophobic/Aromatic: ILE62, PRO136, VAL135, TYR134, LEU188, LEU132, VAL110, ALA83, VAL70, CYS199, PHE67.	Charged negative: ASP133, ASP200. Charged positive: ARG141, LYS85. Polar: THR138 H-bond: ARG141, ILE62, VAL135, ASP133 (2). Hydrophobic/Aromatic: ILE62, PRO136, VAL135, TYR134, LEU188, LEU132, VAL110, ALA83, VAL70, CYS199, PHE67.
NPA014875	Charged negative: GLU137, ASP133, ASP200. Charged positive: ARG141. Polar: THR138, ASN186. H-bond: ARG141, PRO136, VAL135, ASP133. Hydrophobic/Aromatic: ILE62, PRO136, VAL135, THR134, LEU132, VAL110, ALA83, LEU188, CYS199, PHE67,	Charged negative: ASP133, ASP200. Charged positive: LYS85. Polar: THR138, ASN186, GLN185. H-bond: ASP133, ILE62, VAL135, ASN186. Hydrophobic/Aromatic: ILE62, PRO136, VAL135, THR134, LEU132, VAL110, ALA83,

	VAL70.	LEU188, CYS199, PHE67, VAL70.
NPA020257	Charged negative: ASP133, ASP200. Charged positive: ARG141, LYS85, LYS183. Polar: THR138, GLN185, ASN186. H-bond: ASP200, GLN185, ILE62. Hydrophobic/Aromatic: LEU188, ILE62, PRO136, VAL135, TYR134, LEU132, VAL110, ALA83, CYS199, VAL70, PHE67.	Charged negative: GLU137, ASP133, ASP200. Charged positive: ARG141, LYS85. Polar: THR138. H-bond: ASP133, VAL135, PRO136 (2). Hydrophobic/Aromatic: LEU188, ILE62, PRO136, VAL135, TYR134, LEU132, VAL110, ALA83, CYS199, VAL70.
NPA004276	Charged negative: ASP133, ASP200. Charged positive: LYS183. Polar: ASN64, ASN186, GLN185, SER66. H-bond: ASP200, ASP133, SER66. Hydrophobic/Aromatic: PRO136, VAL135, TYR134, LEU132, ALA83, ILE62, VAL110, LEU188, CYS199, PHE67, VAL70. Pi-Pi stacking: PHE67	Charged negative: ASP181, GLU137, ASP200. Charged positive: LYS183, ARG141, LYS85. Polar: THR138, ASN186, GLN185, SER66. H-bond: ASN186, PRO136, SER66. Hydrophobic/Aromatic: PRO136, VAL135, TYR134, LEU132, ALA83, ILE62, LEU188, CYS199, PHE67, VAL70. Pi-Pi stacking: TYR134.
NPA034843	Charged negative: GLU137,	Charged negative: ASP133,

	ASP133, ASP200. Charged positive: ARG141, Polar: THR138, GLN185. H-bond: PRO136, ARG141, ILE62. Hydrophobic/Aromatic: ILE62, VAL70, ALA83, VAL110, LEU132, CYS199, TYR134, VAL135, PRO136, LEU188.	ASP200. Charged positive: LYS183. Polar: THR138, GLN185, ASN1836, ASN64. H-bond: ASP200, GLN185. Hydrophobic/Aromatic: ILE62, VAL70, PHE67, ALA83, VAL110, LEU132, CYS199, TYR134, VAL135, LEU188. Pi-Pi stacking: PHE67.
NPA036064	Charged negative: GLU137, ASP133, ASP200. Charged positive: ARG141. Polar: GLN185, ASN186, ASN64, THR138. H-bond: PRO136, ARG141. Hydrophobic/Aromatic: PRO136, VAL135, TYR134, LEU132, ALA83, VAL110, CYS199, VAL70, PHE67, LEU188, ILE62.	Charged negative: ASP133. Charged positive: ARG141. Polar: GLN185, GLN72. H-bond: ILE62, ASP133. Hydrophobic/Aromatic: PRO136, VAL135, TYR134, LEU132, ALA83, VAL110, CYS199, VAL70, LEU188, ILE62. Pi-cation: ARG141.
7YG_4ACC_REF (Reference)	Charged negative: GLU137, ASP133, ASP200. Charged positive: ARG141, LYS85.	Charged negative: GLU137, ASP133, ASP200. Charged positive: ARG141, LYS85.

	Polar: THR138. H-bond: ASP133, ARG141, LYS85. Hydrophobic/Aromatic: VAL61, ILE62, PRO136, VAL135, TYR134, LEU132, VAL110, CYS199, ALA83, PHE67, VAL70.	Polar: NONE H-bond: ILE62, VAL135, ASP133. Hydrophobic/Aromatic: VAL61, ILE62, PRO136, VAL135, TYR134, LEU132, LEU188, VAL110, CYS199, ALA83, PHE67, VAL70.
AR-A014418	Charged negative: GLU137, ASP133. Charged positive: ARG141, LYS85. Polar: GLN72, THR138. H-bond: PRO136, VAL135 (2). Hydrophobic/Aromatic: ILE62, VAL61, LEU188, ALA83, VAL70, LEU132, VAL110, TYR134, CYS199, VAL135, PRO136.	Charged negative: GLU137, ASP133, ASP200. Charged positive: ARG141, LYS85. Polar: GLN185, THR138. H-bond: ARG141, VAL135 (2). Hydrophobic/Aromatic: ILE62, LEU188, ALA83, VAL70, LEU132, VAL110, TYR134, CYS199, VAL135, MET101, PHE201.
Tideglusib	Charged negative: ASP133, ASP200. Charged positive: LYS85. Polar: ASN64, GLN185, ASN186. H-bond: LYS85 Hydrophobic/Aromatic: PRO136, VAL135, TYR134, VAL110,	Charged negative: ASP133, ASP200. Charged positive: LYS85, ARG141. Polar: THR138, ASN186. H-bond: VAL135. Hydrophobic/Aromatic: PRO136, VAL135, TYR134, LEU132,

	LEU132, ALA83, CYS199, LEU188, VAL70, PHE67, ILE62.	ALA83, CYS199, LEU188, VAL70, ILE62.
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AutoQSAR Modeling and pIC₅₀ Prediction

The predicted pIC₅₀ values of the selected compounds were generated using the Schrödinger AutoQSAR model. The model was built using 1,500 known inhibitors curated from the ChEMBL database and rigorously validated to ensure predictive reliability. The AutoQSAR module of Maestro Schrödinger was used in constructing the QSAR model. AutoQSAR divided the selected compounds into the training (blue dots) and test (red dots) sets, with a random training set at 75% (1,125 compounds) and test set at 25% (375 compounds). The best model (kpls_radial_32) for predicting the pIC₅₀ values of the hit compounds has an R-squared value of 0.7123 and a Q-squared value of 0.6223, the root mean square error (RMSE) stands at 0.7338, with a standard deviation (S.D) of 0.6427 (Supplementary Table 2). The dataset's predictive and observed values are comprehensively detailed in Supplementary Table 2. The optimal model, kpls_radial_32, exhibited reasonable agreement between observed and predicted pIC₅₀ values across training and test sets, as illustrated by the clustering of data points along the diagonal line in Figure 6.

The predicted pIC₅₀ values for the hit compounds and reference inhibitors are presented in Table 6. NPA020257 displayed the highest predicted activity with a pIC₅₀ of 6.8202, followed by AR-A014418 (6.7077), NPA014875 (6.5484), the co-crystallized reference (6.5007), Tideglusib (6.2569), NPA011425 (6.2308), NPA034843 (6.1215), NPA036064 (5.9002), and NPA004276 (5.831).

Table 6: Predicted pIC50 values of the top compounds and reference ligands

NPA ID/Entry name	Pred Y
NPA011425	6.2308
NPA014875	6.5484
NPA020257	6.8202
NPA004276	5.831
NPA034843	6.1215
NPA036064	5.9002
7YG_4ACC_REF (Reference)	6.5007
AR-A014418	6.7077
Tideglusib	6.2569

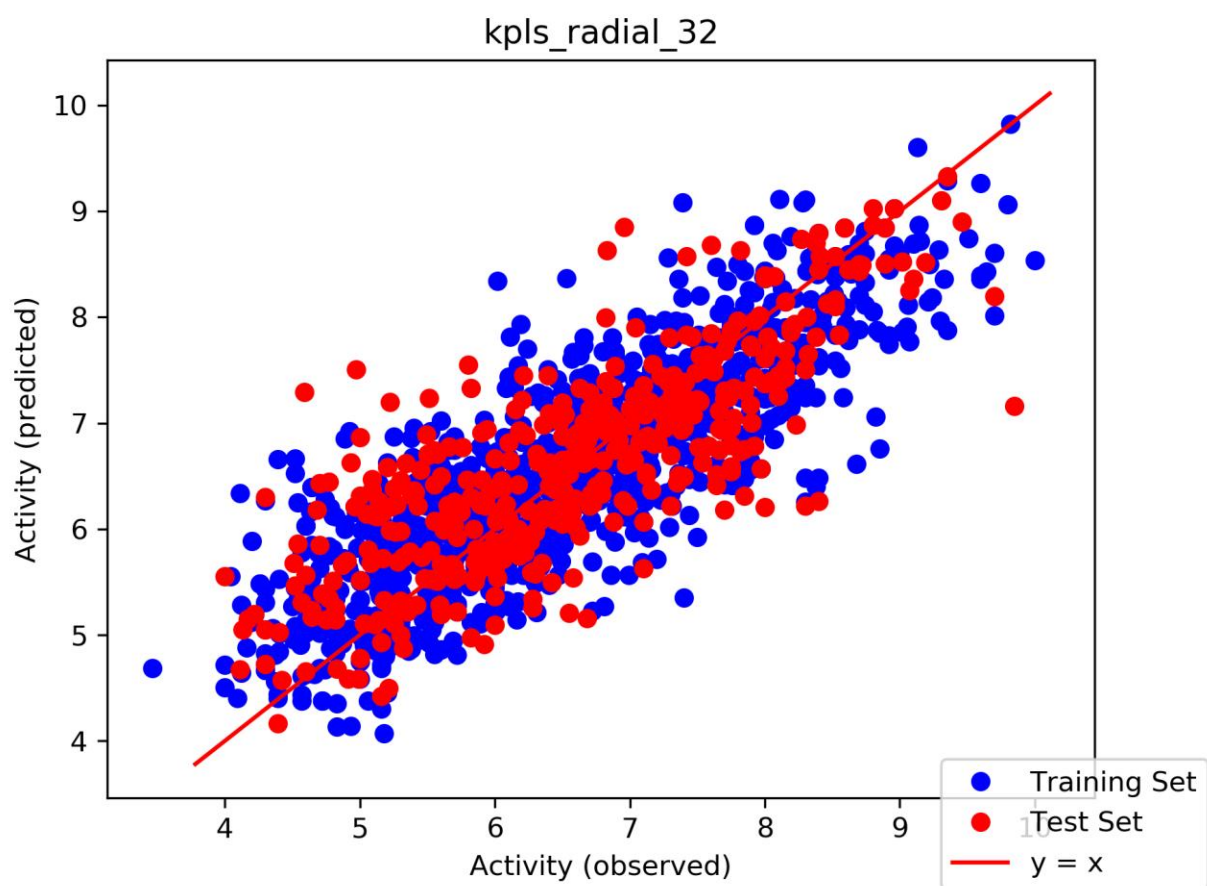


Figure 6: Plot of pIC50 observed vs pIC50 predicted of QSAR model.

ADMET Prediction

The physicochemical, pharmacokinetic, and medicinal chemistry properties of the hit compounds were evaluated using the SwissADME server (Table 7). All six natural compounds complied fully with Lipinski's Rule of Five (zero violations). The molecular weights of the six natural product hits ranged from 308.29 to 430.41 g/mol, while the reference ligand, AR-A014418, Tideglusib had a molecular weight of 441.51, 308.31, and 334.39 g/mol respectively. The hydrogen-bond acceptor counts for all tested compounds ranged from 2 to 8, while the hydrogen-bond donor counts ranged from 0 to 5, falling within the acceptable range. Most compounds showed TPSA values below the 140 Å² threshold associated with good gastrointestinal absorption. NPA020257 (TPSA = 158.4 Å²) and the reference ligand (TPSA = 151.58 Å²) exceeds this threshold, consistent with their prediction of low GI absorption. None of the six natural product hits was predicted to cross the blood–brain barrier. In contrast, Tideglusib was predicted to be BBB permeant. Except for NPA020257 and the co-crystallized ligand, all molecules were predicted to have high GI absorption. All compounds showed similar oral bioavailability scores (0.55–0.56), corresponding to an estimated probability of at least 10% oral bioavailability. All top compounds had a Synthetic accessibility score ≤ 4.61 .

Cytochrome P450 inhibition profiling suggested a generally favorable metabolic profile. None of the tested compounds was predicted to inhibit CYP2C19, except AR-A014418 and Tideglusib, and none inhibited CYP3A4 except the co-crystallized reference ligand. CYP1A2 inhibition was predicted for NPA011425, the co-crystallized reference ligand, AR-A014418, and Tideglusib, whereas NPA014875, NPA020257, NPA004276, NPA034843, and NPA036064 were predicted to be non-inhibitors. With the exception of NPA004276, the reference ligand, and Tideglusib, all compounds were predicted to be non-inhibitors of CYP2C9. CYP2D6 inhibition was predicted for NPA011425, and the reference ligand.

Table 7: Physiochemical, drug-likeness and medicinal properties of the top compounds using SwissADME

NPA ID/Entry name	Physiochemical Properties				Pharmacokinetics							Drug-likeness & Medicinal chemistry		
	MW	H-bond acceptors	H-bond donors	TPSA	GI absorption	BBB permeant	CYP1A2 inhibitor	CYP2C19 inhibitor	CYP2C9 inhibitor	CYP2D6 inhibitor	CYP3A4 inhibitor	Lipinski violations	Bioavailability Score	Synthetic Accessibility
NPA011425	308.29	5	3	99.35	High	No	Yes	No	No	Yes	No	0	0.55	3.09
NPA014875	406.38	7	3	125.04	High	No	No	No	No	No	No	0	0.55	4.61
NPA020257	396.4	6	5	158.4	Low	No	No	No	No	No	No	0	0.55	3.69
NPA004276	430.41	7	4	132.13	High	No	No	No	Yes	No	No	0	0.55	3.82
NPA034843	310.3	5	2	92.42	High	No	No	No	No	No	No	0	0.56	2.75
NPA036064	337.33	5	3	104.45	High	No	No	No	No	No	No	0	0.55	3.52
7YG_4ACC_REF (Reference)	441.51	8	3	151.58	Low	No	Yes	No	Yes	Yes	Yes	0	0.55	3.55
AR-A014418	308.31	5	2	137.31	High	No	Yes	Yes	No	No	No	0	0.55	2.94
Tideglusib	334.39	2	0	72.24	High	Yes	Yes	Yes	Yes	No	No	0	0.55	3.16

7YG_4ACC_REF (Reference)	1800	4	Inactive	Inactive	Active	Inactive	Inactive	Inactive	Inactive
AR-A014418	1350	4	Inactive	Inactive	Inactive	Active	Inactive	Inactive	Active
Tideglusib	1000	4	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive

Active: Compound is predicted to have that type of toxicity

Inactive: Compound is predicted not to have that type of toxicity

The in silico toxicological assessment of the natural hit compounds, co-crystallized ligand and positive inhibitors (AR-A014418, and Tideglusib) are detailed in Table 8. NPA011425 and NPA014875 displayed a lower predicted LD50 (600 mg/kg) relative to other natural hits (ranging from 1250 to 2000mg/kg) and the reference compounds (ranging from 1350 to 1800mg/kg), and all compounds were assigned to toxicity class IV in ProTox-3, suggesting moderate acute oral toxicity. Interesting, all the natural product atlas identified compounds were predicted to be inactive for cardiotoxicity, cytotoxicity, Aryl hydrocarbon Receptor (AhR) and Androgen Receptor (AR). NPA011425 was uniquely predicted to show hepatotoxicity, whereas the remaining natural hits were predicted to be inactive for this organ toxicity. The studied compounds are inactive for cytotoxicity in toxicity end point probability, whereas the native reference ligand is active. AR-A014418 was predicted to be active for Aryl hydrocarbon Receptor (AhR) and Mitochondrial Membrane Potential (MMP) toxicity in Tox21 Nuclear receptor signaling pathways and Tox21 Stress response pathways probabilities respectively. NPA014875 and NPA004276 showed predicted activity for mitochondrial membrane potential (MMP) toxicity, while NPA004276 was additionally flagged for estrogen receptor interaction. Supplementary figure 1 shows the radar chart all molecules toxicity prediction.

DISCUSSION

The synchronized pathogenesis of Type 2 Diabetes (T2D) and Metabolic Dysfunction-Associated Steatohepatitis (MASH) represents a complex, multi-organ clinical challenge centered on insulin resistance, lipotoxicity, and chronic inflammation [52-55]. Glycogen Synthase Kinase-3 beta (GSK-3 β) is a master metabolic regulator that integrates these pathways by directly controlling glycogen synthesis, gluconeogenesis, lipogenesis, and pro-inflammatory signaling [56,57]. Sustained hyperactivation of GSK-3 β under chronic metabolic stress perpetuates peripheral and hepatic insulin resistance, drives steatosis, and promotes hepatic stellate cell (HSC) activation, which leads to progressive liver fibrosis [56,58,59]. Consequently, small-molecule inhibition of GSK-3 β represents a highly promising dual-action therapeutic strategy. In this study, we employed a rigorous, multi-tiered computational pipeline to mine the Natural Products Atlas, successfully identifying six structurally diverse, microbial-derived compounds as promising GSK-3 β inhibitors with favorable drug-like properties.

The initial screening of the 36,545 microbial compounds from the NP Atlas was designed to quickly eliminate non-drug-like space. Lipinski's Rule of Five (RO5) is a well-established filter for estimating oral drug absorption and membrane permeability [60]. In this study, 21,390 compounds (58.5% of the starting library) satisfied the RO5 parameters (zero violations), demonstrating that the NP Atlas is highly enriched with structurally complex yet drug-like microbial secondary metabolites. The subsequent application of a receptor-based e-pharmacophore model acted as a stringent spatial and electronic filter. Pharmacophore modeling identifies the essential spatial arrangement of chemical features required to form a high-affinity complex with a target receptor [61, 62]. Our model, derived from the human GSK-3 β crystal structure (PDB ID: 4ACC) complexed with its co-crystallized ligand, identified four crucial features: three aromatic rings and one hydrogen bond donor. By requiring all four pharmacophoric features to be present, this step successfully eliminated molecules lacking the structural components necessary to form stable interactions with the key residues in the GSK-3 β active site. This significantly reduced the rate of false positives before starting computationally intensive molecular docking.

The reliability of any structure-based virtual screening campaign hinges on the validity of its docking protocol. To this end, we employed a two-pronged validation strategy: re-docking (pose reproduction) and enrichment analysis (active vs. decoy discrimination) [63,64].

Re-docking analysis of the co-crystallized ligand into the GSK-3 β binding pocket yielded an RMSD of 2.2831 Å, slightly above the traditional 2.0 Å threshold. Although this exceeds the conventional cutoff, visual inspection confirmed that the critical pharmacophoric features, particularly the hinge-binding motif, were faithfully reproduced. This phenomenon, where peripheral, solvent-exposed flexible groups inflate the calculated RMSD without compromising functionally relevant binding interactions, is well documented in the docking literature [64, 65].

Enrichment analysis provided robust statistical validation of the discriminatory power of our docking workflow. The ROC value of 0.98 and AUC of 0.97 indicate near-perfect discrimination between active GSK-3 β inhibitors and structurally similar decoys [66, 67]. The Enrichment Factor at 5% (EF_{5%}), demonstrating 100% recovery of active compounds within the top 5% of ranked results, is particularly meaningful as virtual screening campaigns typically advance only the top-scoring fraction of compounds to experimental validation [67]. The Robust Initial

Enhancement (RIE) value of 12.27 and BEDROC values ($\alpha = 8.0$: 0.883; $\alpha = 20.0$: 0.741; $\alpha = 160.9$: 0.341) further confirm the protocol's strength in “early recognition,” the critical ability to rank true actives at the top of the screening hit list [67]. The minimum Tanimoto coefficient of 0.0093 among active ligands further validates that this excellent performance was achieved against a structurally diverse active set, demonstrating that the protocol is not biased toward any single chemical scaffold [68].

The ATP-binding cleft of GSK-3 β is highly conserved, with the hinge region, specifically residues Asp133, Tyr134, and Val135, playing an indispensable role in anchoring competitive inhibitors through directed hydrogen-bonding networks [69–71]. Our molecular interaction analyses revealed that all six natural product hits established robust hydrogen bonds with this critical hinge motif.

Specifically, the top-scoring compound in Extra Precision (XP) docking, NPA011425 (docking score: -11.059 kcal/mol), established a robust bidentate hydrogen-bonding network with Asp133, Val135, and Pro136. Similarly, NPA014875 (docking score: -9.545 kcal/mol) formed a total of four hydrogen bonds with Asp133, Val135, Pro136, and Arg141. Engaging Arg141 is highly significant, as interactions with residues near the catalytic loop can influence kinase active-site stabilization, ligand affinity, and inhibitor selectivity [69, 72]. Beyond the hinge region, the lead natural products engaged deeply with the hydrophobic pocket lined by residues Ile62, Val70, Ala83, Leu132, and Leu188. This hydrophobic enclosure is vital for stabilizing the ligand and reducing desolvation penalties [73].

Crucially, compounds such as NPA004276 and NPA034843 also engaged in pi-pi stacking interactions with Phe67, a key residue within the glycine-rich P-loop, and targeting this region can stabilize ATP-competitive inhibitors within the kinase active site [69, 70, 72]. When compared to the reference clinical candidate Tideglusib, which primarily interacted with Lys85 in our rigid docking models, the natural product hits exhibited much broader and more complex interaction networks within the ATP-binding site, suggesting a potent, competitive mechanism of action.

The observed discrepancy between the initial docking scores and the MMGBSA binding energies, where NPA014875, NPA004276, and NPA020257 exhibited superior stability with

binding energies of -62.67 kcal/mol, -52.49 kcal/mol, and -52.27 kcal/mol respectively despite their initial scores, is a documented phenomenon in computational screening. As noted by Genheden and Ryde [74], docking scores often prioritize geometric fit and hydrogen bonding, whereas MM-GBSA incorporates physics-based force fields to account for desolvation and structural relaxation.

Kinases are highly flexible enzymes, and their active-site loops often undergo significant conformational rearrangements upon ligand binding [75, 76]. IFD analysis demonstrated that these natural products maintain, and in some cases enhance, their interaction networks when receptor backbone and side-chain flexibility are introduced, supporting their ability as potential biological binders.

A robust quantitative structure-activity relationship (QSAR) model was developed to predict the inhibitory activity (pIC_{50}) of compounds against the target. The high predicted potency of NPA020257, despite its moderate docking score (-8.221 kcal/mol), suggests that its overall chemical topology is highly optimized for GSK-3 β inhibition, making it an exceptionally strong candidate for further developmental studies.

A major hurdle in metabolic disease drug discovery is the high rate of clinical failure due to poor pharmacokinetic properties or unacceptable toxicological profiles [77, 78]. We therefore subjected our lead compounds to rigorous *in silico* ADME and toxicity profiling using SwissADME and ProTox-3.0. All six natural product hits exhibited excellent drug-likeness, complying fully with Lipinski's Rule of Five with zero violations. The uniform Bioavailability Scores (0.55–0.56) across all candidates indicate approximately 55% probability of achieving $\geq 10\%$ oral bioavailability, an acceptable threshold for oral drug development [79,80]. Furthermore, they exhibited favorable Synthetic Accessibility (SA) scores (≤ 4.61), suggesting they are amenable to chemical synthesis and structural modification [81]. Good gastrointestinal absorption is strongly correlated with a Topological Polar Surface Area (TPSA) of less than $140 \text{ \AA}^2$ [80, 82]. Our results showed that five of the six natural product hits fall within this ideal range.

A critical finding of our pharmacokinetic analysis is that none of the six natural product hits were predicted to cross the blood-brain barrier (BBB), whereas the clinical control Tideglusib was

predicted to be BBB-permeant. While BBB permeability is essential for CNS indications, such as Alzheimer's disease, where Tideglusib has been evaluated, it represents a major liability for peripheral metabolic indications like T2DM and MASH [83, 84]. Central GSK-3 β inhibition has been associated with neuropsychiatric effects and alteration of circadian rhythms [56,85,86]. Therefore, the absolute peripheral restriction of our six hits is a major therapeutic advantage, minimizing the risk of centrally mediated off-target effects.

The Cytochrome P450 (CYP) superfamily is responsible for the metabolism of approximately 75% of all marketed drugs, with five major isoforms, CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4, collectively metabolizing the majority of clinically administered xenobiotics [87,88]. Inhibition of these enzymes by an investigational compound can precipitate clinically significant drug-drug interactions (DDIs), elevating plasma concentrations of co-administered drugs and increasing the risk of toxicity [87, 89]. This concern is particularly relevant for T2DM and MASH patients, who often present with multiple comorbidities, including cardiovascular disease, dyslipidemia, and hypertension, requiring polypharmacy [90]. NPA014875, NPA020257, NPA034843, and NPA036064 emerged with the cleanest CYP profiles, showing no inhibition across any of the five major CYP isoforms tested. This pristine metabolic profile substantially reduces DDI risk and represents a significant clinical advantage for these candidates [79, 87, 89]. Conversely, the reference compounds present multi-enzyme CYP inhibition profiles. The predicted enzyme-inhibitory profiles for NPA011425 and NPA004276 warrant lead optimization efforts to mitigate these liabilities.

The ProTox-3.0 toxicity analysis evaluated multiple parameters relevant to drug safety, including acute oral toxicity (LD₅₀), organ toxicities (hepatotoxicity, cardiotoxicity), cytotoxicity, and key Tox21 endpoints related to nuclear receptor signaling and stress response pathways. The Globally Harmonized System (GHS) classifies chemical toxicity into six categories based on LD₅₀ values, with Class I (LD₅₀ $\leq$ 5 mg/kg) being the most toxic and Class VI (LD₅₀ > 5000 mg/kg) being non-toxic [91]. All studied compounds (natural product hits and reference inhibitors) were classified as Class IV (LD₅₀: 300–2000 mg/kg), considered “slightly toxic” or “harmful if swallowed.” NPA011425 and NPA014875 exhibited the lowest LD₅₀ values (600 mg/kg), while NPA004276, NPA034843, and NPA036064 demonstrated the most favorable LD₅₀ values (2000 mg/kg), outperforming all reference compounds. These toxicity predictions

and class assignments support continued prioritization of the safer candidates for subsequent experimental validation [92,93].

The Aryl Hydrocarbon Receptor (AhR) is a ligand-activated transcription factor of the basic helix-loop-helix Per-Arnt-Sim (bHLH-PAS) family, traditionally recognized for its role in mediating responses to environmental toxicants such as dioxins and polycyclic aromatic hydrocarbons [94,95]. AhR activation by xenobiotics induces phase I and phase II drug-metabolizing enzymes, notably CYP1A1 and CYP1B1, which can generate reactive metabolites capable of damaging DNA and other macromolecules [96]. Moreover, AhR activation has been implicated in immunotoxicity, carcinogenesis, and metabolic dysregulation [95,97]. Encouragingly, all six natural product hits were predicted to be inactive against AhR, indicating low risk for activating this pro-toxicant pathway. In contrast, AR-A014418 was predicted to be AhR-active, representing a notable safety liability for this reference compound.

The Androgen Receptor (AR) is a nuclear hormone receptor that regulates the expression of genes involved in male sexual differentiation, reproduction, and various metabolic processes [98]. Activation or antagonism of AR by xenobiotics can cause endocrine disruption, leading to reproductive toxicity, infertility, and developmental abnormalities [99]. All six natural product hits and reference compounds were predicted to be inactive at AR, indicating low risk for androgenic endocrine disruption—a particularly important consideration for chronic therapeutic agents intended for long-term use in metabolic disease management.

The Estrogen Receptor Alpha (ER α) is a nuclear hormone receptor that mediates the effects of estrogens on reproductive tissues, bone metabolism, cardiovascular function, and the central nervous system [100]. Inappropriate activation of ER α by xenobiotics (xenoestrogens) is associated with increased risk of hormone-dependent cancers, including breast, endometrial, and ovarian cancers, as well as reproductive abnormalities and metabolic disorders [99,101,102]. Among the natural product hits, only NPA004276 was predicted to be ER α -active, while all other hits and reference compounds showed no ER α activity. This finding represents a significant developmental concern for NPA004276 and would necessitate further investigation or structural modification to eliminate this estrogenic liability, particularly given the increased risk of estrogen-sensitive malignancies in obese and metabolic disease populations [102,103].

The Mitochondrial Membrane Potential (MMP) is a fundamental parameter of mitochondrial function, generated by the electron transport chain and essential for ATP synthesis, calcium homeostasis, and apoptotic regulation [104]. Compounds that disrupt MMP can precipitate mitochondrial dysfunction, leading to cellular ATP depletion, oxidative stress, and triggering of the intrinsic apoptotic pathway [104,105]. Drug-induced mitochondrial toxicity is a leading cause of post-marketing drug withdrawals, with notable examples including troglitazone, cerivastatin, and nefazodone [105, 106]. MMP disruption is particularly relevant for compounds intended to treat liver diseases such as MASH, where pre-existing hepatocyte mitochondrial dysfunction is a hallmark pathological feature [107,108]. NPA014875 and NPA004276 were predicted to be MMP-active, along with the reference inhibitor AR-A014418. This finding represents a meaningful concern for these compounds, particularly in the context of MASH where hepatic mitochondrial reserve is already compromised, and lead optimization strategies may be warranted for these candidates.

Drug-induced liver injury (DILI) remains a major cause of acute liver failure and is an important contributor to drug attrition and post-marketing safety restrictions [109,110]. For compounds intended to treat MASH, hepatotoxicity assessment is of paramount importance, as any inherent hepatotoxic liability could exacerbate the underlying liver pathology rather than ameliorate it [52,53,109]. NPA011425 was the only natural product hit predicted to be hepatotoxic, presenting a paradoxical safety challenge given its superior binding score but intended use in liver disease. This finding necessitates careful consideration of NPA011425's developmental pathway. Importantly, all six natural product hits were predicted to be non-cytotoxic and non-cardiotoxic, indicating low risk for general cellular damage and cardiac dysfunction, a significant advantage over many kinase inhibitors that exhibit cardiotoxic liabilities [111]. Notably, the co-crystallized reference ligand itself was flagged as cytotoxic, further highlighting the favorable safety profile of our natural product hits.

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