Cell. Mol. Biol. (ISSN: 1165-158X)

Journal Homepage: www.cellmolbiol.org

Cellular and Molecular Biology

Original Article

In-silico and in-vitro studies revealed alpha-amyrin as a potent inhibitor of TLR2 for the therapeutics of bacterial infection and sepsis

Thoraya A-Elgadir[1], Basiouny El-Gamal[1*], Mohamed Abd Ellatif[1,2], Khalid Ali Nasif[1,3], Safaa Omer[1], Muniera Mohieldeen[1], Ayyub A. Patel[1], Mohammed Amanullah[1], Arshi Malik[4], Ahmed A. Mahfouz[5,6], Ayman H. Shaamash[7] Awad S. Alsamghan[5]

[1] Department of Clinical Biochemistry, College of Medicine, King Khalid University, Abha, Saudi Arabia
[2] Department of Medical Biochemistry, Faculty of Medicine, Mansoura University, Mansoura, Egypt
[3] Department of Medical Biochemistry, Faculty of Medicine, Minia University, Minia, Egypt
[4] CQRL BITS LLP, Chennai, India
[5] Department of Family and Community Medicine, King Khalid University, Abha, Saudi Arabia
[6] Department of Epidemiology, High Institute of Public Health, Alexandria University, Egypt
[7] Department of Obstetrics and Gynecology, College of Medicine, King Khalid University, Abha, Saudi Arabia

Article Info

Article history:

Received: January 21, 2024
Accepted: April 07, 2024
Published: June 30, 2024

Use your device to scan and read the article online

Abstract

This study employed a multifaceted approach to investigate the inhibitory potential of alpha-amyrin against TLR2, a key player in bacterial infection and sepsis. A high-resolution TLR2 model was constructed using Swiss-MODEL, exhibiting excellent quality with 100% sequence identity and coverage. Cavity detection revealed five significant cavities on TLR2. Molecular docking identifies alpha-amyrin as a potent inhibitor, displaying a strong binding affinity of -8.6 kcal/mol. Comprehensive analyses, including ADMET predictions, PASS analysis, and SwissTargetPrediction, affirm alpha-amyrin's drug-like properties and diverse biological activities. Cytotoxicity assays on HEK-293 cells confirm its safety, and fluorescence-based inhibition assays provide empirical evidence of its inhibitory potency on TLR2 enzymatic activity. Further validations in HUVECs show a significant decrease in TLR2 mRNA expression ($p<0.01$) and activity ($p<0.05$) upon alpha-amyrin treatment. In conclusion, this integrative study positions alpha-amyrin as a promising therapeutic candidate for TLR2 inhibition, emphasizing its potential in combating bacterial infections with safety and efficacy.

Keywords: Alpha-amyrin, bacterial infection, HEK-293, HUVECs, Sepsis, Toll like receptor 2 (TLR2).

1. Introduction

Toll-like receptors (TLRs) are a crucial component of the innate immune system, serving as sentinels that recognize various microbial components and activate immune responses [1]. Among them, Toll-like receptor-2 (TLR-2) has emerged as a key player in recognizing bacterial cell wall components and initiating immune responses [2]. This receptor holds immense clinical significance due to its involvement in the pathogenesis of bacterial infections and sepsis [3,4].

TLR-2 is a transmembrane protein belonging to the Toll-like receptor family, characterized by an extracellular domain containing leucine-rich repeats (LRRs) responsible for pathogen recognition. TLR-2 forms heterodimers with either TLR-1 or TLR-6 to recognize a diverse range of microbial components, particularly lipoproteins and lipopeptides derived from bacterial cell walls [5]. Upon ligand binding, TLR-2 heterodimers initiate signaling cascades through the recruitment of adaptor proteins, such as MyD88 (myeloid differentiation primary response 88), ultimately leading to the activation of nuclear factor-kappa B (NF-κB) and the production of pro-inflammatory cytokines, chemokines, and antimicrobial peptides [6, 7] This orchestrated response is essential for the clearance of invading pathogens and the initiation of adaptive immune responses. While this response is crucial for pathogen clearance, dysregulated TLR2 activation can contribute to the development of systemic inflammatory conditions, such as sepsis [8]. Bacterial infections, particularly those leading to severe conditions such as sepsis, pose a significant global health threat.

Sepsis, a life-threatening condition, stands as a formidable challenge within the realm of healthcare, demanding a comprehensive understanding of its intricate patho-

* Corresponding author.
E-mail address: basiouny_el_gamal@hotmail.com (B. El-Gamal).

Doi: http://dx.doi.org/10.14715/cmb/2024.70.6.16

physiology, timely intervention, and ongoing research efforts. This complex syndrome arises when the body's response to an infection becomes dysregulated, leading to a cascade of inflammatory processes that can result in organ dysfunction and, if left untreated, death [9, 10]. At its core, sepsis begins as a response to infection. The immune system, activated by the presence of pathogens, initiates a series of signalling pathways aimed at eradicating the invaders [11, 12]. However, in sepsis, this response spirals out of control, triggering a systemic inflammatory response syndrome that can lead to widespread tissue damage [13]. Key players in the pathophysiology of sepsis include pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukins, as well as anti-inflammatory mediators attempting to counterbalance the excessive inflammation [10, 14,15]. The delicate equilibrium between pro- and anti-inflammatory signals becomes disrupted, tipping the scales toward a state of hyperinflammation, endothelial dysfunction, and microcirculatory abnormalities [16, 17].

Sepsis arises when the body's immune response to an infection becomes uncontrolled, leading to widespread inflammation and organ dysfunction [10,11,12]. TLR2, by recognizing specific molecular patterns on bacterial pathogens, plays a pivotal role in triggering the immune response [8]. However, excessive TLR2 activation has been implicated in the pathogenesis of sepsis, contributing to the overwhelming inflammation and tissue damage characteristic of the condition [18]. The rationale behind therapeutic TLR2 inhibition in sepsis lies in the need to strike a delicate balance between an effective immune response and the prevention of collateral tissue damage. By selectively modulating TLR2 activity, it becomes possible to temper the inflammatory cascade without compromising the overall ability of the immune system to combat the underlying infection [8]. Despite the considerable potential, challenges persist in the development of TLR2 inhibitors.

Achieving selectivity and minimizing off-target effects remain critical considerations. Additionally, the complex nature of the immune response demands a nuanced understanding of the temporal and spatial aspects of TLR2 modulation to optimize therapeutic outcomes. The inhibition of TLR2 holds significant promise for the development of therapeutics aimed at treating bacterial infections and preventing the progression to sepsis [8]. Small molecule inhibitors offer the advantage of precise targeting, enabling the modulation of TLR2 activity without completely suppressing the immune response [19, 20, 21]. This fine-tuned approach could mitigate the risk of immunosuppression often associated with broad-spectrum anti-inflammatory therapies. Moreover, TLR2 inhibition could represent a valuable adjunctive therapy to conventional antibiotic treatment. By attenuating the host inflammatory response, small molecule inhibitors may help prevent the development of sepsis-associated complications, such as organ failure and tissue damage [22, 23].

This study aimed to identify the small molecule inhibitors against TLR2 for the therapeutics of bacterial infection, and sepsis. Because rapid initiation of pro-inflammatory responses by TLR2 is critical in controlling infection, however, dysregulation of this signaling can lead to excessive release of pro-inflammatory cytokines. Therefore, inhibiting TLR2 activation can help in curbing an overactive immune response and avoid damaging immunopathology.

2. Material and methods

2.1. Protein structure homology modelling and preparation of TLR2

Homology modelling is a widely used technique to predict the three-dimensional structure of a protein based on its homologous proteins with known structures. SWISS-MODEL is a popular web-based tool for protein structure homology modelling (https://swissmodel.expasy.org/) [24, 25, 26, 27, 28] The 3D structure of the target protein, TLR2, was modelled using Swiss-Model. Briefly, the FASTA sequence of TLR2 was obtained from PubMed. The template sequences of homologous proteins with known three-dimensional structures were searched using Protein Data Bank (PDB). Thereafter, we selected one suitable template structure (PDB ID: 1FYW) based on sequence similarity, structure quality, and biological relevance. This template structure was used to model the structure of TLR2 using SWISS-MODEL [24, 25, 27, 28, 29]. High-resolution structure of TLR2 was retrieved from the Swiss-MODEL server (Fig. 1) and visualized using PyMOL and Chimera to analyse the structural features and properties. Preparing the receptor for molecular docking is a crucial step, standard receptor preparation protocol was followed to refine the structure of TLR2 [30, 31 The structure of TLR2 was inspected for any steric clashes, missing atoms, or unusual bond angles. The structure was assigned partial charges and atom types to the protein atoms using force field parameters suitable for molecular docking. The structure was finally saved in PDBQT format, a format compatible with molecular docking.

2.2. Selection and preparation of ligands for molecular docking with TLR2

Ligand molecules were obtained from NPACT (Naturally occurring Plant-based Anticancerous Compound-Activity-Target DataBase) [32]. NPACT is a curated database of plant-derived natural compounds that exhibit anti-cancerous activity. It contains 1574 entries and each record provides information on the structure, properties, and drug likeness of compounds. The SDF format files of ligands were downloaded from PubChem. The structures were checked and bond lengths, bond angles, and torsional angles were corrected if any. Finally, the structures of ligands were converted to PDBQT format, a format compatible with molecular docking.

2.3. Cavity detection on TLR2

Structure-based cavity detection on TLR2 was conducted using CB-Dock2 (http://cadd.labshare.cn/cb-dock2/.). CB-Dock is a protein-ligand docking method that automatically identifies the binding sites, calculates the center and size, customizes the docking box size according to the query ligands and then performs the molecular docking with AutoDock Vina [33]. The TLR2 file in PDB format was uploaded to the CB-Dock 2 server. Parameters such as specifying the probe radius, cavity size, etc., were opted for cavity detection.

2.4. Molecular docking

Docking calculations were carried out using DockingServer [34]. Gasteiger partial charges were added to the ligand atoms. Non-polar hydrogen atoms were merged, and rotatable bonds were defined. Docking calculations were carried out on alpha-amyrin:TLR2 model. Essential

hydrogen atoms, Kollman united atom type charges, and solvation parameters were added with the aid of AutoDock tools [35]. Affinity (grid) maps of 40×40×40 Å (x, y, and z) grid points and 0.375 Å spacing were generated using the Autogrid program [35. AutoDock parameter set- and distance-dependent dielectric functions were used in the calculation of the van der Waals and the electrostatic terms, respectively. Docking simulations were performed using the Lamarckian genetic algorithm (LGA) and the Solis & Wets local search method [36. The initial position, orientation, and torsions of the ligand molecules were set randomly. All rotatable torsions were released during docking. Each docking experiment was derived from 2 different runs that were set to terminate after a maximum of 250000 energy evaluations. The population size was set to 150. During the search, a translational step of 0.2 Å, and quaternion and torsion steps of 5 were applied.

Additionally, the protein-ligand complex was reduced using CB-Dock2 to validate the binding affinity of protein-ligand complex [33].

2.5. Assessment of ADMET properties of alpha-amyrin

ADMET properties were assessed using pkCSM (Pharmacokinetics and Chemistry of Small Molecules) online tool (https://biosig.unimelb.edu.au/pkcsm/). This tool predicts pharmacokinetic and toxicity properties of small molecules, including ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) properties. The lead molecule in SMILES format was uploaded to pkCSM online tool to determine the ADMET properties.

2.6. Prediction of Activity Spectra for alpha-amyrin using PASS online and Swiss Target Prediction tool

In the realm of chemoinformatics and drug discovery, the prediction of activity spectra for ligand is an important step. Current study employed PASS online (http://www.pharmaexpert.ru/passonline/) and Swiss Target Prediction tool (http://www.swisstargetprediction.ch/) to determine the biological activity spectrum associated with the lead molecule. PASS (Prediction of Activity Spectra for Substances) Online is a web-based platform that provides predictions for the biological and pharmacological activities of chemical compounds. It is a tool designed to assist researchers in identifying potential activities and properties of small molecules. The predictions generated by PASS are based on the analysis of chemical structures and their relationships to known activities from a vast database. SwissTargetPrediction is a web-based tool developed to predict potential targets for small molecules or drugs. It is designed to facilitate the identification of proteins that may interact with a given compound. This information can be valuable in drug discovery and understanding the potential effects of a compound in biological systems. The SMILES notation of lead molecules was uploaded to PASS online and SwissTargetPrediction tool to determine the biological activity spectrum of lead molecules.

2.7. Preparation of test compound HEK-293 cell line

Alpha-amyrin (purity ≥98.0% HPLC) was acquired from Sigma, USA (CAS Number: 638-95-9) and dissolved in DMSO (Sigma, USA) to create a stock solution at 1 Mg/mL concentration. The HEK-293 cell line, obtained from ATCC, was cultured in DMEM media supplemented with 10% FBS and 1% antibiotics.

2.8. Cell culture and cytotoxicity assay

HEK-293 cells were cultured in a 96-well microplate with 5,000 cells per well in 100 µL of DMEM medium. After a 24-hour incubation at 37°C with 5% humidity, cells were treated with various alpha-amyrin concentrations (0 to 1 Mg) for 72 hours. The culture medium was replaced with 100 µL of fresh medium containing 0.5 mg/mL MTT reagent for a 4-hour incubation. After aspirating the MTT-containing medium, 100 µL of DMSO was added to dissolve formazan crystals through gentle agitation for 10 minutes. Absorbance at 570 nm was measured using a plate reader. Net absorbance was calculated by subtracting values from blank wells. Cell viability was determined by dividing net absorbance of test wells by that of control wells (untreated cells) and multiplying by 100. Additionally, a time-dependent cell viability assay (24, 48, and 72 hours) was conducted to assess the impact of IC50 concentration of alpha-amyrin on HEK-293 cells.

2.9. *In vitro* inhibition assay

The TLR2 protein (Cat: 10061-H08B) with purity > 85%, obtained from SinoBiological, China, was used in fluorescence binding studies with alpha-amyrin. A JASCO 6300 spectrofluorometer equipped with a 1 cm quartz cell was employed. Fluorescence spectra were generated by exciting TLR2 at 280 nm and recording emission spectra from 300 to 500 nm. Excitation and emission slit widths were set at 10 mm, with a medium sensitivity response setting. The acquired fluorescence data for both the TLR2 and the TLR2:alpha-amyrin complex were analysed to extract binding parameters. This analysis aimed to reveal the inhibitory effects of alpha-amyrin on TLR2.

2.10. mRNA expression and activity of TLR2 in alpha-amyrin-treated HUVECS

HUVECs were treated with the IC_{50} concentration of alpha-amyrin for 48 hours. RNA was extracted using TRIzol™ Reagent (Ambion, Carlsbad, CA, USA), followed by cDNA synthesis with the Verso cDNA Synthesis Kit (Thermo Fisher Scientific, USA). Quantitative qRT-PCR using SYBR™ Green Master Mix (Thermo Fisher Scientific, USA) on an Applied Biosystems RT-PCR System assessed TLR2 expression, normalized to the housekeeping gene β-actin. The $2^{-\Delta\Delta CT}$ method calculated the relative fold change. Primers were adapted from Krivan et al. 2019. The primer sequences employed were as follows; TLR-2: forward 5'-TGGGCTGACTTCTCTCAATG-3' and reverse 5'-TTCATCGGTGAGCTGACTTC-3' (reverse) and β-actin: forward 5'-CCAGAATGAGGATCCCAGAA-3' and reverse 5'-ACCACCTGAAACATGCAACA-3'. Further, TLR2 activity in HUVECs lysates was quantified using the Human TLR-2 ELISA Kit from Elabsciences following the protocol as described [37].

2.11. Statistical analysis

The data was analysed with the help of available tool SPSS (ver. 22). Cytotoxicity assay & mRNA expression was collective from three times different experiments. The data is designed as mean ± SD. The t-test was used to match the mean values between the two groups. The $p<0.05$ was reflected statistically important.

3. Results

3.1 Protein structure homology modelling and preparation of TLR2

Figure 1A represents the high-resolution structure of TLR2 model downloaded from the Swiss-MODEL. Seq Identity and coverage for the model was 100%. The quality of the model was very good and quality assessment showed GMQE score of 0.89 and QMEANDisCo Global score of 0.82 ± 0.07. GMQE and QMEANDisCo global give an overall model quality measurement between 0 and 1, with higher numbers indicating higher expected quality. GMQE is coverage dependent, i.e. a model covering only half of the target sequence is unlikely to get a score above 0.5. QMEANDisCo on the other hand evaluates the model 'as is' without explicit coverage dependency. Graphical representation of QMEANDisCo local quality estimate is presented in Figure 1B.

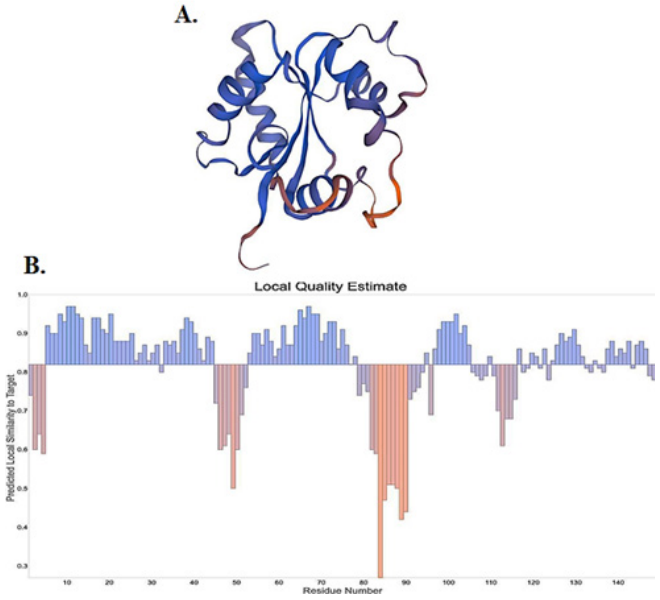


Fig. 1. (A) High-resolution structure of TLR2 model downloaded from the Swiss MODEL. **(B)** The "Local Quality" plot shows, for each residue of the model (reported on the x-axis), the expected similarity to the native structure (y-axis). Typically, residues showing a score below 0.6 are expected to be of low quality.

3.2 Cavity detection on TLR2

The key findings regarding cavity detection of TLR2 are presented in Table 1, representing the number of cavities identified in the target protein using CB-Dock2. Five major cavities (C1-C5) were detected. Detailed information on the size, volume, or other relevant characteristics of the detected cavities is presented in Table 1. Cavity 1 (C1) showed a maximum Cavity Size of X, Y, and Z (19, 24, 18), and Cavity Volume (Å3) of 2395. Cavity 2 (C2) showed a Cavity Size of X, Y, and Z (8, 8, 3), and Cavity Volume (Å3) of 141. Cavity 3 (C3) showed a Cavity Size of X, Y, and Z (7, 7, 5), and Cavity Volume (Å3) of 114. Cavity 3 (C3) showed a Cavity Size of X, Y, and Z (5, 7, 6), and Cavity Volume (Å3) of 113. Cavity 5 (C5) showed a maximum Cavity Size of X, Y, and Z (4, 5, 6), and Cavity Volume (Å3) of 65. Fig. 2a, 2b, 2c, 2d, and 2e represents the cavities C1, C2, C3, C4, and C5 of the TLR2, respectively.

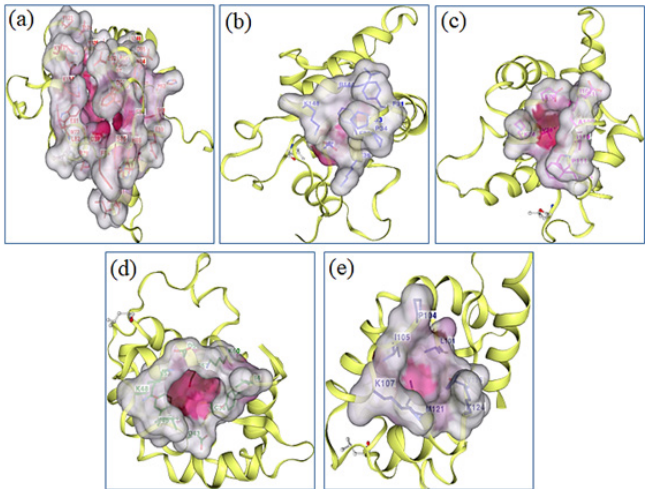


Fig. 2. a-e represent the cavities C1, C2, C3, C4, and C5 of the TLR2, respectively.

3.3. Molecular docking revealed alpha-amyrin as potential inhibitor of TLR2

From the screening of ligands of NPACT database against TLR2, the ligand alpha-amyrin showed highest fitness of 99.98% and highest predicted binding affinity. The alpha-amyrin (Fig. 3a) was selected as the top hit and docked against the target protein TLR2. The molecular docking results of the TLR2: alpha-amyrin complex are visually represented in Fig. 3a-d. The docking analysis yielded affinity scores and docked poses, revealing that alpha-amyrin exhibited a notable binding affinity score of -8.6 kcal/mol with TLR2. Fig. 3b represents the cartoon presentation of TLR2: alpha-amyrin complex. Fig. 3c presents the 2D plot represents the amino acid residues of TLR2 making different interactions with alpha-amyrin. Fig. 3d represents the surface view of TLR2:alpha-amyrin complex. The docking pose exhibited the successful docking of alpha-amyrin into the deep binding cavity of TLR2. Alpha-amyrin formed several polar, hydrophobic and other types of the interactions with TLR2 (Table 2). Further the interaction of alpha-amyrin with the amino acid resi-

Table 1. Detailed information on the size, volume, or other relevant characteristics of the detected cavities (C1-C5).

CurPocket ID	Cavity volume (Å^3)	Center (x, y, z)	Cavity size (x, y, z)
C1	2395	-2, 93, 23	19, 24, 18
C2	141	-14, 77, 17	8, 8, 3
C3	114	11, 97, 10	7, 7, 5
C4	113	-16, 95, 13	5, 7, 6
C5	65	14, 85, 16	4, 5, 6

Table 2. Decomposed Interaction Energies in kcal/mol. Alpha-amyrin formed several polar, hydrophobic and other types of the interactions with TLR2

Polar	Hydrophobic	Other
LYS119 (-0.1612)	TRP77 (-5.5388)	THR64 (-0.9336)
	PHE87 (-3.6082)	LYS63 (-0.8027)
	ALA97 (-0.7267)	LYS116 (-0.1581)
	LEU99 (0.313)	ASP91 (8.2765)
	PHE66 (0.9478)	
	ILE120 (1.5257)	
	ILE58 (8.3317)	

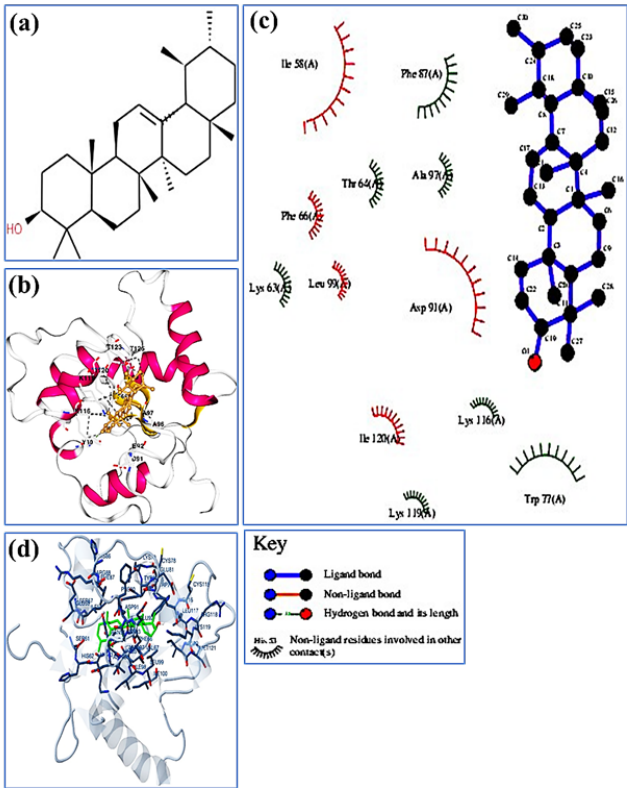


Fig. 3. Structure of alpha-amyrin and molecular docking results of the TLR2:alpha-amyrin complex. (a) Structure of the ligand alpha-amyrin; (b) represents the cartoon presentation of TLR2: alpha-amyrin complex; © presents the 2D plot represents the amino acidresidues of TLR2 making different interactions with alpha-amyrin; (d) represents the surface view of TLR2:alpha-amyrin complex.

dues of TLR2 was also confirmed by the HBPlot analysis of alpha-amyrin:TLR2 complex (Fig. 4).

3.4. ADMET predictions, PASS analysis, and SwissTargetPrediction

ADMET predictions, PASS analysis, and SwissTargetPrediction revealed that alpha-amyrin has drug-like properties. The outcomes of ADMET predictions are summarized in Table 3. ADMET assessments indicated that alpha-amyrin meets essential pharmacokinetic criteria, positioning it as a promising candidate for therapeutic development targeting TLR2 inhibition against bacterial infections.

PASS Analysis results (Table 4) revealed favourable biological properties associated with alpha-amyrin, emphasizing its relevance in various significant biological processes.

SwissTargetPrediction analysis affirms and elaborates

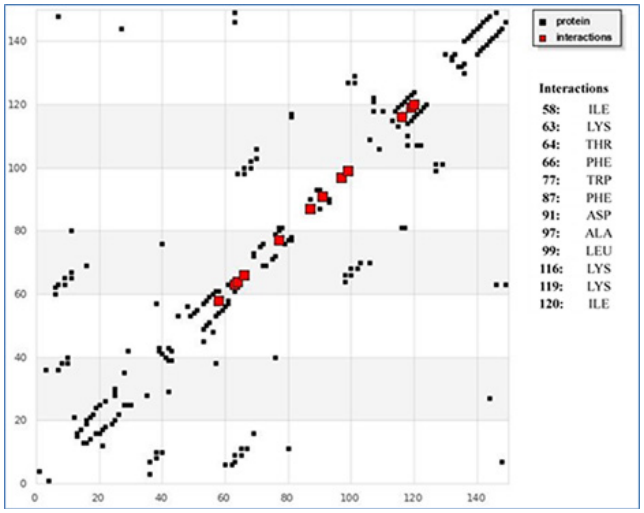


Fig. 4. HBPlot of TLR2: alpha-amyrin complex representing interactions.

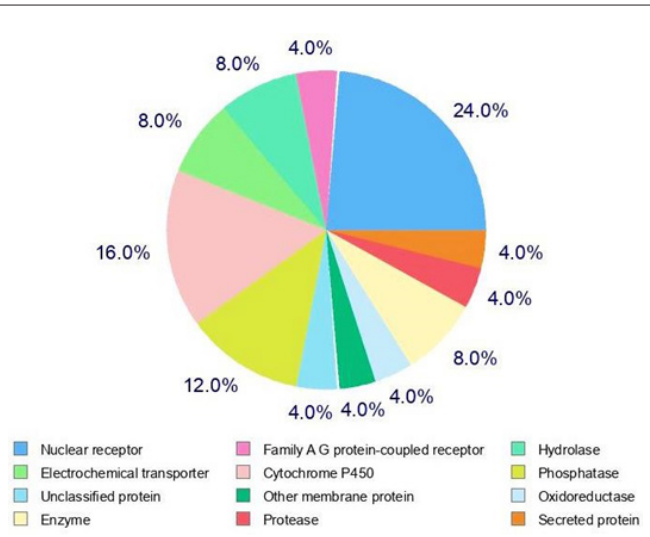


Fig. 5. SwissTargetPrediction analysis. This figure represents the association of alpha-amyrin withdifferent biological properties.

on the diverse biological properties linked to alpha-amyrin, as illustrated in Fig. 5.

3.5. Concentration-dependent cytotoxicity assay of alpha-amyrin and Time-dependent

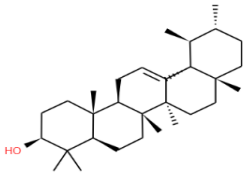
The results of concentration-dependent cell toxicity assay are presented in Fig. 6a. This assay showed no significant effect of alpha-amyrin on the viability of HEK-293 cells. Further, the IC_{50} value for alpha-amyrin was found to be 93.14 µM.

The results of time-dependent cell toxicity assay are

Table 3. In silico prediction of ADME & Toxicity properties for alpha-amyrin (AA).

	Properties												
	Absorption	**Distribution**			**Metabolism**							**Excretion**	**Toxicity**
Models	Intestinal absorption (human)	VDss (human)	BBB permeability	CNS permeability	CYP Substrate		Inhibitor					Total clearance	AMES toxicity /Hepatotoxicity
					2D6	3A4	1A2	2C19	2C9	2D6	3A4		
Unity	Numeric (% absorbed)	Numeric (log L/kg)	Numeric (Log BB)	Numeric (Log PS)	Categorical (yes/no)							Numeric (log ml/min / kg)	Categorical (yes/no)
Predicted values													
AA	94.062	0.266	0.674	-1.773	NO	Yes	NO	NO	NO	NO	NO	0.119	NO

Table 4. PASS (Prediction of Activity Spectra for Substances) analysis of alpha-amyrin. Probability "to be active" was set at Pa>0,7.

Compound	Structure	Pa	Pi	Activity
Alpha-amyrin		0,934	0,002	Insulin promoter
		0,926	0,002	Hepatoprotectant
		0,911	0,004	Apoptosis agonist
		0,901	0,005	Antineoplastic
		0,897	0,002	Transcription factor NF kappa B stimulant
		0,897	0,002	Transcription factor stimulant
		0,890	0,003	Chemopreventive
		0,889	0,004	Antiinflammatory
		0,885	0,003	Oxidoreductase inhibitor
		0,878	0,003	Antiprotozoal (Leishmania)
		0,876	0,003	Hepatic disorders treatment
		0,864	0,004	Caspase 3 stimulant
		0,865	0,007	Mucomembranous protector
		0,851	0,005	Hypolipemic
		0,840	0,003	Antiulcerative
		0,839	0,005	Membrane integrity antagonist
		0,835	0,002	Nitric oxide antagonist
		0,826	0,019	Testosterone 17beta-dehydrogenase (NADP+) inhibitor
		0,808	0,003	Lipid peroxidase inhibitor
		0,809	0,017	Alkenylglycerophosphocholine hydrolase inhibitor
		0,793	0,003	Antiviral (Influenza)
		0,789	0,002	Caspase 8 stimulant
		0,788	0,002	Antinociceptive
		0,781	0,011	Alkylacetylglycerophosphatase inhibitor
		0,773	0,004	Phosphatase inhibitor
		0,772	0,003	Wound healing agent
		0,782	0,015	Acylcarnitine hydrolase inhibitor
		0,753	0,001	DNA ligase (ATP) inhibitor
		0,755	0,006	Antisecretoric
		0,731	0,004	Gastrin inhibitor
		0,756	0,033	CYP2J substrate
		0,716	0,005	Antineoplastic (lung cancer)

Pa - Probability "to be active"; Pi - Probability "to be inactive"

presented in Fig. 6b. Results showed that the increasing exposure time (24 hrs, 48 hrs, and 72 hrs) of alpha-amyrin did not exert any significant effect on the viability of HEK-293 cells.

3.6 Fluorescence-based inhibition assay and

The fluorescence emission spectra of TLR2, acquired at different alpha-amyrin concentrations spanning from 0 to 24 µM, are illustrated in Fig. 7. Examination of the data disclosed a consistent trend: with the elevation of alpha-amyrin concentration, a concurrent decline in the fluorescence emitted by TLR2 was observed. This empirical evidence strongly supports the assertion that alpha-amyrin functions as an inhibitor of TLR2, impeding its enzymatic activity. These findings unmistakably establish a pronounced reduction in the activity of TLR2 as a consequence of escalating alpha-amyrin concentrations, thereby underscoring its inhibitory potency on TLR2.

3.7 mRNA expression and activity of TLR2 in alpha-amyrin-treated HUVECs

The findings regarding mRNA expression and TLR2 activity are depicted in Fig. 8a & 8b, respectively. The results demonstrated a significant decrease in both mRNA expression ($p < 0.01$) and activity ($p < 0.05$) of TLR2 in

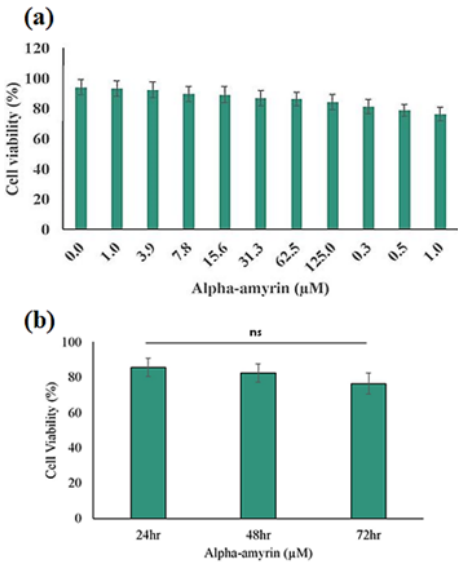


Fig. 6. (a) Viability of HEK-293 treated cells with different concentrations of alpha-amyrin (0 to1M) for 24 hrs. (b) Viability of HEK-293 treated cellswith IC_{50} concentration of alpha amyrin (93.14 µM) for 24, 48, and 72 hrs. The data are presented as mean ± SD of three independent experiments. ANOVA was employed to compare the mean values. The $p<0.05$ was considered as statistically significant. ns; nonsignificant.

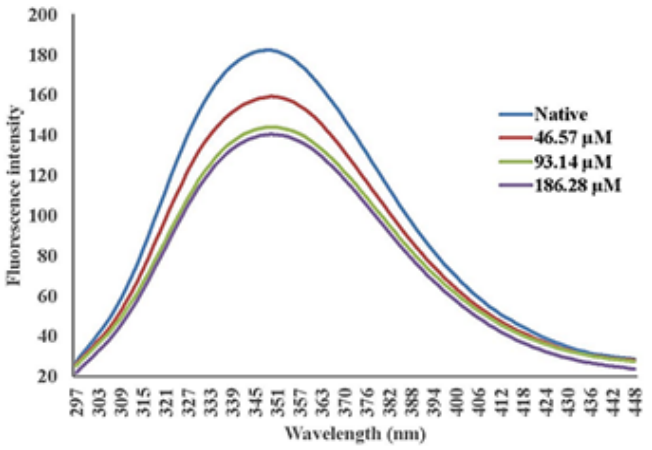


Fig. 7. Fluorescence emission spectra for both native TLR2 and TLR2 treated with varying concentrations of alpha-amyrin IC_{50}, half of IC_{50}, anddouble of the IC_{50}). An effective concentration of 93.14 µM was identified for inhibiting TLR2 activity.

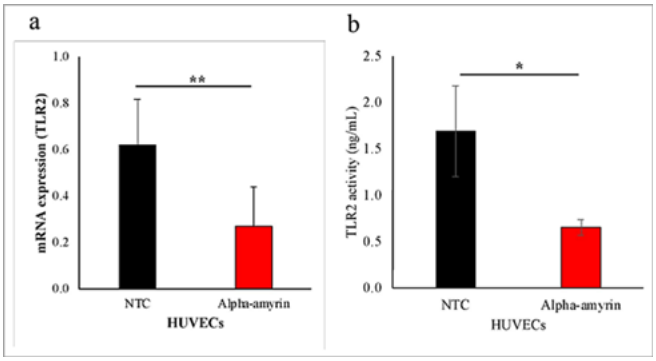


Fig. 8. Effect of IC_{50} dose of alpha-amyrin on the mRNA expression **(a)** and activity **(b)** of TLR2 in HUVECs. * $p<0.05$, ** $p<0.01$.

HUVECs subjected to alpha-amyrin treatment compared to untreated cells. This observation signifies the inhibitory impact of alpha-amyrin on TLR2.

4. Discussion

Considering the role of TLR2 in bacterial infection and sepsis, there is ongoing research to develop therapeutics targeting TLR2. These may include TLR2 modulators or inhibitors to regulate the immune response and prevent excessive inflammation [8]. However, it's important to note that the modulation of TLR2 should be carefully balanced, as an overly suppressed immune response could compromise the ability to fight off infections. TLR2 is a crucial component of the immune system, playing a pivotal role in the recognition of bacterial pathogens [18]. Its activation contributes to the initiation of immune responses against infections, but dysregulated TLR2 activation can be implicated in the pathogenesis of sepsis [38]. Understanding these mechanisms holds potential for the development of targeted therapeutics for bacterial infections and sepsis.

This study employed a comprehensive approach integrating protein homology modelling, molecular docking, and experimental validation of alpha-amyrin as a potent inhibitor of TLR2. This integrative analysis provides a comprehensive understanding of the inhibitory effects of alpha-amyrin on TLR2, highlighting its potential as a therapeutic candidate against bacterial infections. The study begins with the generation of a high-resolution TLR2 model using Swiss-MODEL. SWISS-MODEL is extensively used to predict the three-dimensional structures of proteins for which experimental structures are not available. It is a powerful tool in structural bioinformatics, providing researchers with a reliable and automated platform for predicting protein structures [24, 25, 27]. Its applications range from fundamental research to practical applications in drug discovery and protein engineering [26, 39, 40]. The model of TLR2 exhibited high quality with a Seq Identity and coverage of 100%, GMQE score of 0.89, and QMEANDisCo Global score of 0.82 ± 0.07. The model's coverage-dependent and coverage-independent assessments ensure its reliability. Cavity detection revealed five major cavities in TLR2. Further, the study predicted alpha-amyrin as a potential inhibitor of TLR2 based on its high fitness and predicted binding affinity. Cavity 1 on the TLR2 was targeted for molecular docking with alpha-amyrin. Molecular docking is a computational technique integral to drug design, facilitating the exploration of interactions between small molecules and target proteins [31]. It predicts the binding orientation and affinity of a ligand within a protein's active site, aiding in the identification of potential drug candidates (Chen et al., 2023). By simulating and analyzing molecular interactions, docking guides the rational design of pharmaceuticals, optimizing their efficacy and specificity [30]. This approach accelerates drug discovery by narrowing down the pool of compounds for experimental validation, ultimately fostering the development of novel and targeted therapeutic agents for various diseases [41]. In this study, molecular docking revealed a strong binding affinity score of -8.6 kcal/mol, indicating a robust interaction between alpha-amyrin and TLR2. This indicates that alpha-amyrin is a potent inhibitor of TLR2. The docking poses and interactions showed alpha-amyrin's successful docking into the deep binding cavity of TLR2, forming various polar and hydrophobic interactions.

Molecular docking aids in predicting binding affinities, guiding the selection of lead compounds with favorable drug-like characteristics. Evaluating drug-like properties and biological activity spectrum of predicted inhibitor molecules is a pivotal step in drug discovery [42]. Properties such as absorption, distribution, metabolism, excretion, and toxicity are crucial determinants of a drug's efficacy and safety [43]. In the current study, analysis of alpha-amyrin's drug-like properties involves ADMET predictions, PASS analysis, and SwissTargetPrediction. Results indicate that alpha-amyrin possesses favorable pharmacokinetic characteristics, making it a promising therapeutic candidate for TLR2 inhibition against bacterial infections. PASS and SwissTargetPrediction analyses highlight additional biological properties associated with alpha-amyrin, further supporting its potential as a multifaceted therapeutic agent.

Cytotoxicity assays play a vital role in screening and identifying inhibitor molecules for drug development [44]. These assays assess the toxicity of potential inhibitors on living cells, providing crucial insights into their safety profiles [45]. By gauging the impact on cell viability and function, researchers can eliminate cytotoxic compounds early in the screening process. This contributes to the selection of lead molecules with optimal therapeutic potential and

minimal harm to normal cells. Cytotoxicity assays thus enhance the efficiency of drug discovery, ensuring that candidate inhibitors progress with a higher likelihood of successful development and reduced risks of adverse effects in subsequent stages. In this study, concentration-dependent and time-dependent cytotoxicity assays on HEK-293 cells demonstrate that alpha-amyrin exhibits no significant cytotoxic effects within the tested concentration range (up to 93.14 μM) and exposure times (24, 48, and 72 hours). These findings suggest that alpha-amyrin holds promise as a safe therapeutic agent. Fluorescence-based inhibition assays are invaluable in screening and identifying inhibitor molecules during drug discovery [46]. These assays exploit changes in fluorescent signals to detect alterations in enzymatic or molecular activity upon inhibitor binding [47, 48, 49, 50]. The method provides real-time, sensitive, and high-throughput analysis, enabling rapid identification of potential inhibitors. The fluorescence-based inhibition assay provided empirical evidence of alpha-amyrin's inhibitory potency on TLR2. The gradual decline in TLR2 fluorescence with increasing alpha-amyrin concentration strongly supports its role as an inhibitor, hindering TLR2 enzymatic activity. By measuring fluorescence changes, we gained insights into the inhibitory potency and specificity of alpha-amyrin. The study extends to human umbilical vein endothelial cells (HUVECs) to evaluate the impact of alpha-amyrin on TLR2 at the molecular level. Both mRNA expression and activity of TLR2 showed a significant decrease in alpha-amyrin-treated HUVECs compared to untreated cells. This observation underscores the inhibitory influence of alpha-amyrin on TLR2 and its potential therapeutic relevance in modulating immune responses. Taken together, this comprehensive analysis positions alpha-amyrin as a promising multifaceted therapeutic agent against bacterial infections, emphasizing its potential for further drug development.

5. Conclusion

Targeting TLR2 emerges as a promising avenue for bacterial infection and sepsis therapeutics. This study elucidates the inhibitory potential of alpha-amyrin on TLR2 through a rigorous combination of computational modeling, molecular docking, and experimental validations. The multifaceted approach employed in this research not only indicates alpha-amyrin as a predicted promising TLR2 inhibitor but also provides valuable insights into its safety profile and potential therapeutic applications. Further exploration of alpha-amyrin as a novel therapeutic agent against bacterial infections, leveraging its inhibitory effects on TLR2, warrants additional investigations. In conclusion, this study paves the way for developing small molecule inhibitors against TLR2, offering a multifaceted approach to combat bacterial infections and sepsis, with implications for future clinical applications.

Acknowledgements

The authors extend their appreciation to the Deanship of Scientific Research at King Khalid University for funding this work through Large Group Research Project under grant number (R.G.P 2 / 210 / 1444)**.**

Consent for publications

The author approved the final manuscript for publication.

Ethics approval and consent to participate

The present research is not used human or animals.

Informed consent

The authors declare not used any patients in this research.

Author's contribution

TAE: helped the design the experiments and to draft manuscript primary. BEG: Helped to design the experiment to write the final draft. MAE: contributed to enzymatic assays and revision of manuscript. SO: Edit manuscript, MS: Helped to design the docking work. KAN: contributed to the analysis, and editing manuscript. AM: helped to edit the manuscript. MA: helped in design of experiments. AAP: read the manuscript and make suitable editing. AS: helped in revision. AM: helped in statistical analysis and ASA: finalised final draft after revision.

References

1. Lester SN, & Li K (2014) Toll-like receptors in antiviral innate immunity. J Mol Biol 426(6): 1246-1264. https://doi.org/10.1016/j.jmb.2013.11.024.
2. Sameer AS, & Nissar S (2021) Toll-Like Receptors (TLRs): Structure, Functions, Signaling, and Role of Their Polymorphisms in Colorectal Cancer Susceptibility. Biomed Res Int 1157023. https://doi.org/10.1155/2021/1157023.
3. Gong Z, Zhang J, Zhang S, Cao J, Fu Y, Hu X, Zhao J, Gu B, Li Q, Zhang K, Ren P, Liu B, & Mao W. (2022) TLR2, TLR4, and NLRP3 mediated the balance between host immune-driven resistance and tolerance in *Staphylococcus aureus*-infected mice. Microb Pathog169, 10567. https://doi.org/10.1016/j.micpath.2022.105671.
4. Lima CX, Souza DG, Amaral FA, Fagundes CT, Rodrigues IP, Alves-Filho JC, Kosco-Vilbois M, Ferlin W, Shang L, Elson G, & Teixeira MM. (2015) Therapeutic Effects of Treatment with Anti-TLR2 and Anti-TLR4 Monoclonal Antibodies in Polymicrobial Sepsis. PloS One 10(7): e0132336. https://doi.org/10.1371/journal.pone.0132336.
5. Trinchieri G, & Sher A. (2007) Cooperation of Toll-like receptor signals in innate immune defence. Nat Rev Immunol 7(3): 179-190. https://doi.org/10.1038/nri2038.
6. Duan T, Du Y, Xing C, Wang HY, & Wang RF. (2022) Toll-Like Receptor Signaling and Its Role in Cell-Mediated Immunity. Front Immunol 13, 812774. https://doi.org/10.3389/fimmu.2022.812774.
7. Nie L, Cai SY, Shao JZ, & Chen J. (2018) Toll-Like Receptors, Associated Biological Roles, and Signaling Networks in Non-Mammals. Front Immunol 9, 1523. https://doi.org/10.3389/fimmu.2018.01523.
8. Simpson ME, & Petri WA, Jr (2020) TLR2 as a Therapeutic Target in Bacterial Infection. Trends Mol Med 26(8): 715-717. https://doi.org/10.1016/j.molmed.2020.05.006.
9. Gyawali B, Ramakrishna K, & Dhamoon AS. (2019) Sepsis: The evolution in definition, pathophysiology, and management. SAGE Open Med 7, 2050312119835043. https://doi.org/10.1177/2050312119835043.
10. Jarczak D, Kluge S, & Nierhaus A. (2021) Sepsis-Pathophysiology and Therapeutic Concepts.Front Med 8, 628302. https://doi.org/10.3389/fmed.2021.628302.
11. Delano MJ, & Ward PA. (2016) The immune system's role in sepsis progression, resolution, and long-term outcome. Immunol Rev 274(1): 330-353. https://doi.org/10.1111/imr.12499.
12. Hotchkiss RS, Moldawer LL, Opal SM, Reinhart K, Turnbull IR,

& Vincent JL. (2016) Sepsis and septic shock. Nat Rev Dis Primers 16045. https://doi.org/10.1038/nrdp.2016.45.
13. Chakraborty RK & Burns B. (2023) Systemic Inflammatory Response Syndrome. In Stat Pearls. Stat Pearls Publishing.
14. Huang M, Cai S, & Su J. (2019) The Pathogenesis of Sepsis and Potential Therapeutic Targets. Int J Mol Sci 20(21): 5376. https://doi.org/10.3390/ijms20215376.
15. Schulte W, Bernhagen J, & Bucala R. (2013) Cytokines in sepsis: potent immunoregulators and potential therapeutic targets--an updated view. Mediators of Inflamm 165974. https://doi.org/10.1155/2013/165974.
16. Arora J, Mendelson AA, & Fox-Robichaud A. (2023) Sepsis: network pathophysiology and implications for early diagnosis. Am J Physiol Regul Integr Comp Physiol 324(5): R613-R624. https://doi.org/10.1152/ajpregu.00003.2023.
17. Joffre J, Hellman J, Ince C, & Ait-Oufella H. (2020) Endothelial Responses in Sepsis. Am J Respir Crit Care Med 202(3): 361-370. https://doi.org/10.1164/rccm.201910-1911TR.
18. Oliveira-Nascimento L, Massari P, & Wetzler LM. (2012) The Role of TLR2 in Infection and Immunity. Front Immunol 3, 79. https://doi.org/10.3389/fimmu.2012.00079.
19. Hennessy EJ, Parker AE, & O'Neill LA. (2010) Targeting Toll-like receptors: emerging therapeutics? Nat Rev Drug Discov 9(4): 293-307. https://doi.org/10.1038/nrd3203
20. Li H, Liu S, Han J, Li S, Gao X, Wang M, Zhu J, & Jin T. (2021) Role of Toll-Like Receptors in Neuroimmune Diseases: Therapeutic Targets and Problems. Front Immunol 12, 777606. https://doi.org/10.3389/fimmu.2021.777606.
21. O'Neill LA, Bryant CE, & Doyle SL. (2009) Therapeutic targeting of Toll-like receptors for infectious and inflammatory diseases and cancer. Pharmacol Rev 61(2): 177-197. https://doi.org/10.1124/pr.109.001073.
22. Crouser E, Exline M, Knoell D, & Wewers MD. (2008) Sepsis: links between pathogen sensing and organ damage. Curr Pharm Des 14(19): 1840–1852. https://doi.org/10.2174/138161208784980572.
23. Zhang YY, & Ning BT. (2021) Signaling pathways and intervention therapies in sepsis. Signal Transduct Target Ther 6(1): 407. https://doi.org/10.1038/s41392-021-00816-9.
24. Bienert S, Waterhouse A, de Beer TA, Tauriello G, Studer G, Bordoli L, & Schwede T. (2017) The SWISS-MODEL Repository-new features and functionality. Nucleic Acids Res 45(D1): D313-D319. https://doi.org/10.1093/nar/gkw1132.
25. Bienert S, Waterhouse A, de Beer TAP, Tauriello G, Studer G, Bordoli L, Schwede T. (2017) The SWISS-MODEL Repository - new features and functionality. Nucleic Acids Res 45 (D1): D313-D319.
26. Bertoni M, Kiefer F, Biasini M, Bordoli L, Schwede T. (2017) Modeling protein quaternary structure of homo- and hetero-oligomers beyond binary interactions by homology Sci Rep 5;7(1):10480.
27. Waterhouse A, Bertoni M, Bienert S, Studer G, Tauriello G, Gumienny R, Heer FT, de Beer TAP, Rempfer C, Bordoli L, Lepore R, Schwede T. (2018) SWISS-MODEL: homology modelling of protein structures and complexes. Nucleic Acids Res 46(W1): W296-W303.
28. Waterhouse A, Bertoni M, Bienert S, Studer G, Tauriello G, Gumienny R, Heer FT, de Beer TAP, Rempfer C, Bordoli L, Lepore R, & Schwede T. (2018) SWISS-MODEL: homology modelling of protein structures and complexes. Nucleic acids Res 46(W1): W296-W303. https://doi.org/10.1093/nar/gky427.
29. Xu Y., Tao X, Shen B, Horng T, Medzhitov R, Manley JL, & Tong L. (2000) Structural basis for signal transduction by the Toll/interleukin-1 receptor domains. Nature 408(6808): 111-115. https://doi.org/10.1038/35040600.
30. Sultan A, Ali R, Ishrat R, & Ali S. (2022). Anti-HIV and anti-HCV small molecule protease inhibitors in-silico repurposing against SARS-CoV-2 Mpro for the treatment of COVID-19. J Biomol Struct Dyn 40(23): 12848-12862. https://doi.org/10.1080/07391102.2021.1979097
31. Sultan A, Ali R, Sultan T, Ali S, Khan NJ, & Parganiha A. (2021). Circadian clock modulating small molecules repurposing as inhibitors of SARS-CoV-2 Mpro for pharmacological interventions in COVID-19 pandemic. Chronobiol Int 38(7): 971-985. https://doi.org/10.1080/07420528.2021.1903027.
32. Mangal M, Sagar P, Singh H, Raghava GP, & Agarwal SM. (2013) NPACT: Naturally Occurring Plant-based Anti-cancer Compound-Activity-Target database. Nucleic Acids Res 41(Database issue), D1124-D1129. https://doi.org/10.1093/nar/gks1047.
33. Liu Y, Grimm M, Dai WT, Hou MC, Xiao ZX, & Cao Y. (2020) CB-Dock: a web server for cavity detection-guided protein-ligand blind docking. Acta Pharmacol Sin 41(1): 138-144. https://doi.org/10.1038/s41401-019-0228-6.
34. Bikadi Z, & Hazai E. (2009) Application of the PM6 semi-empirical method to modeling proteins enhances docking accuracy of AutoDock. J Cheminform 1, 15. https://doi.org/10.1186/1758-2946-1-15.
35. Morris GM, Goodsell DS, et al. (1998) Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function. J Comput Chem 19 (14): 1639-1662.
36. Solis FJ & Wets RJB. (1981) Minimization by Random Search Techniques. Mathematics of Operations Research 6 (1): 19-30 (1981).
37. Sepehri Z, Kiani Z, Nasiri AA, & Kohan F. (2016) Toll-like receptor 2 and type 2 diabetes. Cell Mol Biol Lett 21, 2. https://doi.org/10.1186/s11658-016-0002-4.
38. Kumar V. (2020) Toll-like receptors in sepsis-associated cytokine storm and their endogenous negative regulators as future immunomodulatory targets. Int Immunopharmacol 89(Pt B): 107087. https://doi.org/10.1016/j.intimp.2020.107087.
39. Studer G, Rempfer C, Waterhouse AM, Gumienny G, Haas J, Schwede T. (2020). QMEANDisCo - distance constraints applied on model quality estimation. Bioinformatics 1;36(6):1765-1771.
40. Studer G, Tauriello G, Bienert S, Biasini M, Johner N, Schwede T. (2021) ProMod3 - A versatile homology modelling toolbox. PLOS Comp. Biol 17(1), e1008667.
41. Sahu M, Sultan A, & Barik MR. (2016) Molecular docking and high throughput screening of designed potent inhibitor to PTPN11 involved in Peptic Ulcer. South Asian J Exp Biol 6(4): 124-130. https://doi.org/10.38150/sajeb.6(4).p124-130.
42. Chen X, Xue B, Wahab S, Sultan A, Khalid M, & Yang S. (2023) Structure-based molecular docking and molecular dynamics simulations study for the identification of dipeptidyl peptidase 4 inhibitors in type 2 diabetes. J Biomol Struct Dyn 1-14. Advance online publication. https://doi.org/10.1080/07391102.2023.2291831.
43. Stielow M, Witczyńska A, Kubryń N, Fijałkowski Ł, Nowaczyk J, & Nowaczyk A. (2023) The Bioavailability of Drugs-The Current State of Knowledge. Molecules (Basel, Switzerland) 28(24): 8038. https://doi.org/10.3390/molecules28248038.
44. Szymański P, Markowicz M, & Mikiciuk-Olasik E. (2012) Adaptation of high-throughput screening in drug discovery-toxicological screening tests. Int J Mol Sci 13(1): 427-452. https://doi.org/10.3390/ijms13010427.
45. Lage OM, Ramos MC, Calisto R, Almeida E, Vasconcelos V, & Vicente F. (2018) Current Screening Methodologies in Drug Discovery for Selected Human Diseases. Mar drugs 16(8): 279. https://doi.org/10.3390/md16080279.

46. Kumar V, Chunchagatta Lakshman PK, Prasad TK, Manjunath K, Bairy S, Vasu AS, Ganavi B, Jasti S, & Kamariah N. (2023) Target-based drug discovery: Applications of fluorescence techniques in high throughput and fragment-based screening. Heliyon 10(1): e23864. https://doi.org/10.1016/j.heliyon.2023.e23864.
47. Lea WA, & Simeonov A. (2011) Fluorescence polarization assays in small molecule screening. Expert Opin Drug Discov 6(1): 17-32. https://doi.org/10.1517/17460441.2011.537322.
48. Amini S, Omidi AH, Afkhami H et al. Frequency of aac (6′)-le-aph (2″) gene and resistance to Aminoglycoside antibiotics in Staphylococcus aureus isolates. Cell. Mol. Biomed. Rep. 2024;4(2):120-128. https://doi: 10.55705/cmbr.2023.411361.1166.
49. Long Y, Du X, Ouyang Z et al. Research progress on therapeutic effect and mechanism of hydrocortisone on sepsis. Cell. Mol. Biomed. Rep. 2023;3(3):122-129. doi: https://doi.org/10.55705/cmbr.2023.377524.1090.
50. Alavi M, Rai M, Martinez F et al. The efficiency of metal, metal oxide, and metalloid nanoparticles against cancer cells and bacterial pathogens: different mechanisms of action. Cell. Mol. Biomed. Rep. 2022;2(1):10-21. doi: Https://doi.org/10.55705/cmbr.2022.147090.1023.