Identification of Putative Drug Targets in Highly Resistant Gram-Negative Bacteria; and Drug Discovery Against Glycyl-tRNA Synthetase as a New Target

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ABSTRACT

BACKGROUND: Gram-negative bacterial infections are on the rise due to the high prevalence of multidrug-resistant bacteria, and efforts must be made to identify novel drug targets and then new antibiotics.

METHODS: In the upstream part, we retrieved the genome sequences of 4 highly resistant Gram-negative bacteria (e.g., *Acinetobacter baumannii, Klebsiella pneumoniae, Pseudomonas aeruginosa*, and *Enterobacter cloacae*). The core proteins were assessed to find common, cytoplasmic, and essential proteins with no similarity to the human proteome. Novel drug targets were identified using DrugBank, and their sequence conservancy was evaluated. Protein Data Bank files and STRING interaction networks were assessed. Finally, the aminoacylation cavity of glycyl-tRNA synthetase (GlyQ) was virtually screened to identify novel inhibitors using AutoDock Vina and the StreptomeDB library. Ligands with high binding affinity were clustered, and then the pharmacokinetics properties of therapeutic agents were investigated.

RESULTS: A total of 6 common proteins (e.g., RP-L28, RP-L30, RP-S20, RP-S21, Rnt, and GlyQ) were selected as novel and widespread drug targets against highly resistant Gram-negative superbugs based on different criteria. In the downstream analysis, virtual screening revealed that Rimocidin, Flavofungin, Chaxamycin, 11,11′-O-dimethyl-14′-deethyl-14′-methylelaiophylin, and Platensimycin were promising hit compounds against GlyQ protein. Finally, 11,11′-O-dimethyl-14′-deethyl-14′-methylelaiophylin was identified as the best potential inhibitor of GlyQ protein. This compound showed high absorption capacity in the human intestine.

CONCLUSION: The results of this study provide 6 common putative new drug targets against 4 highly resistant and Gram-negative bacteria. Moreover, we presented 5 different hit compounds against GlyQ protein as a novel therapeutic target. However, further in vitro and in vivo studies are needed to explore the bactericidal effects of proposed hit compounds against these superbugs.

KEYWORDS: Gram-negative bacteria, GlyQ protein, StreptomeDB library, computer-aided drug discovery, virtual screening

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Introduction

Antibiotic therapy is one of the most important approaches of modern medicine to confront infections. The "golden era" of antibiotics ended quickly as researchers could not keep up with the pace of antibiotic discovery in facing emerging-resistant microorganisms. The continued failure to develop or discover new antimicrobial agents and the irrational use of antibiotics in health systems are the predisposing factors for the emergence of antibiotic resistance.[1] Nowadays, numerous major organizations, including the Centers for Disease Control and Prevention (CDC), the Infectious Diseases Society of America (IDSA), the World Economic Forum (WEF), and the World Health Organization (WHO), have declared that antibiotic resistance is a "global public health crisis." The World Health Assembly (WHA) has called on the WHO to propose a global action plan to address the problem of antibiotic resistance.[2]

The spread of antibiotic resistance among Gram-negative bacteria, such as *Acinetobacter baumannii, Klebsiella pneumoniae, Pseudomonas aeruginosa*, and *Enterobacter cloacae*, has become a global crisis.[3] Gram-negative bacteria lead to severe infectious diseases in humans, especially in immunocompromised individuals. Antibiotic-resistant Gram-negative bacteria are responsible for most cases of catheter-related bloodstream infections, ventilator-associated pneumonia, and intensive care unit (ICU)-acquired sepsis. Several mechanisms are involved in the antimicrobial resistance of these bacteria, e.g., expression of antibiotic-inactivating enzymes, intrinsic resistance mechanisms, and chromosomal mutations and acquired mobile

genetic elements carrying resistance genes or non-enzymatic mechanisms.[4,5] Therefore, efforts must be made to develop new strategies for discovering new classes of antibiotics to successfully cure Gram-negative bacterial infections. In this regard, it is crucial to find common and novel drug targets against these bacteria and introduce new agents that specifically affect them, with minor side effects on the host.

The discovery of bioactive molecules by conventional methods is expensive and time-consuming. Currently, new strategies are constantly being sought to optimize this process. Virtual screening is one of the latest approaches for the identification of potentially bioactive molecules. Modern drug discovery, driven by advances in bioinformatics and computational modeling, has enabled virtual screening of biologically active compounds to identify hits and lead compounds.[6,7] Moreover, whole-genome sequencing of bacterial pathogens has facilitated the recognition of new therapeutic targets. Besides, several techniques such as high-throughput protein purification, crystallography, and nuclear magnetic resonance (NMR) spectroscopy have been developed to facilitate a better understanding of the structural details of proteins and protein–ligand complexes.[8] These advances enable computational strategies to penetrate all aspects of drug discovery. Taken together, this knowledge enables the study of the biological effects of a wide range of ligands on a variety of macromolecular targets.[9]

In this regard, this study aimed to find novel and broad-spectrum putative drug targets against 4 highly antibiotic-resistant Gram-negative bacteria (e.g., *A. baumannii, K. pneumoniae, P. aeruginosa*, and *E. cloacae*) by determining the core cytoplasmic proteins of these pathogens. In addition, we focused on glycyl-tRNA synthetase (GlyQ) as a novel essential drug target to introduce several hit compounds from the StreptomeDB library using a computer-aided drug discovery approach. Glycyl-tRNA synthetase is a class II aminoacyl-tRNA synthetase that catalyzes the synthesis of glycyl-tRNA, which is required to insert glycine into proteins. Moreover, in a side reaction, this enzyme also synthesizes di-nucleoside polyphosphates, which are likely to participate in the regulation of cell functions.[10] The results of this study might help confronting drug-resistant infections by proposing new and common drug targets against these superbugs.

Materials and Methods

This study consisted of 2 distinct parts: (1) upstream evaluation and (2) downstream analysis. In the upstream part, we determined the core proteins of the 4 highly antimicrobial-resistant Gram-negative bacteria, including *A. baumannii, K. pneumoniae, P. aeruginosa*, and *E. cloacae*, to find novel putative drug targets using subtractive genomic methods. However, in the downstream section, we focused on GlyQ protein as an essential and common putative drug target and introduced several hit compounds against it using virtual screening approaches.

Upstream analysis

Sequence retrieval and pan/core-proteome analysis. Overall, 4 Gram-negative bacterial agents were selected to identify common and novel drug targets. We chose strains with high prevalent sequence types (STs) of selected strains as follows: *A. baumannii* [ST2] (GenBank Accession No. NZ_CP031380), *P. aeruginosa* [ST235] (GenBank Accession No. NZ_CP029605), *K. pneumoniae* [ST23] (GenBank Accession No. NZ_CP026021), and *E. cloacae* [ST513] (GenBank Accession No. NZ_CP020089). The genomes of these strains were retrieved from the GenBank database.[11] In the next step, the core proteome of the 4 above-mentioned strains was obtained using the Bacterial Pan Genome Analysis pipeline (BPGA) software.[12] Then, clustering was performed by the USEARCH algorithm, using a sequence identity and coverage cut-off ≥ 0.5.

Identification of cytoplasmic and essential proteins. The localization of proteins was investigated by the PSORTb database.[13] Cytoplasmic-localized proteins were compared with the essential genes in the database of Essential Genes, DEG, using BLASTp with a cut-off ≥ 0.8.[14]

Sequence similarity of drug targets to the human proteome. Position-Specific Iterative Basic Local Alignment Search Tool (PSI-BLAST) was used to evaluate the sequence similarity of the essential cytoplasmic proteins to the *Homo sapiens* (Taxid: 9606) with the defined threshold (coverage $\geq 30\%$ and identity $\geq 25\%$).[15] Proteins with similarity to human proteomes were excluded from this study.

Identification of pathogen-specific metabolome proteins. Non-homologous proteins were subjected to the Kyoto Encyclopedia of Genes and Genomes (KEGG) automatic annotation server (KAAS).[16] KEGG is a web-based tool that can analyze a set of genes by online BLAST and assign these genes to known metabolic pathways of an organism based on BLAST similarities.[17] The metabolic pathways of the host and pathogen were compared to identify unique pathways that exclusively belonged to the selected bacterial strains (absent in human metabolic pathways). The proteins belonging to the unique metabolic pathways of the Gram-negative bacterial strains were selected for further analysis.

Evaluation of druggable proteins and introduction to novel drug targets. To identify proteins resembling targets of Food and Drug Administration (FDA)-approved and experimental drugs, the amino acid sequences of proteins were submitted to the DrugBank database and druggable proteins were determined. DrugBank database is a comprehensive drug reference that provides practical information on FDA-approved and experimental drugs, drug interactions, and drug targets.[18] Proteins that showed no similarity to the registered targets in the DrugBank database were considered novel drug targets.

Sequence conservancy of novel drug targets among 4 Gram-negative bacterial strains. The proteins were retrieved from *E. coli, A. baumannii, P. aeruginosa, K. pneumoniae*, and *E. cloacae* using BLASTp. Then multiple sequence alignment was performed using MEGA 11 software[19] and the conservation of proteins was assessed by the ConSurf web server.[20]

Protein data bank availability. The relevant protein data bank (PDB) files of the novel drug targets were investigated using the PDB database at the NCBI BLASTp tool,[21] with coverage ⩾ 80% and identity ⩾ 50%.

Analysis of protein–protein interaction network. The interactions of each novel drug target with other proteins were determined using the STRING database. This database integrates all well-known and predicted physical and functional associations between proteins.[22]

Downstream analysis

Homology modeling and multiple sequence alignment in 1D and 3D layers. Multiple sequence alignment of GlyQ was performed in 1D and 3D layers using Jalview software and the mTM-align server,[23] respectively. The FASTA sequences of GlyQ (glycyl-tRNA synthetase) protein belonging to the 4 mentioned Gram-negative bacteria were submitted to trRosetta for 3D structure prediction. The trRosetta is an algorithm for rapid and accurate prediction of a protein's 3D structure.[24] The quality of each predicted structure was checked using QMEAN and the ProSA-web server.[25] In addition, further 3D structural alignment was performed for *E. coli* GlyQ protein and human glycyl-tRNA synthetase (PDB: 2Q5H) in favor of non-reactivity with human.

Receptor structure information and determination of binding site cavity. The heterotetrameric glycyl-tRNA synthetase has 2 alpha subunits (chains: A and B) and 2 beta subunits (chains: C and D) with glycyl-tRNA aminoacylation activity. In this study, we targeted the aminoacylation cavity of the GlyQ protein (Chain A) to find putative hit compounds against it. For this purpose, we used the software MOLE 2.5 to detect the GlyQ aminoacylation cavity and its boundary residues. MOLE 2.5 is an advanced software tool designed for molecular channel and pore analysis.[26]

Preparation of receptor and determination of grid box for docking studies. The crystallographic structure of the GlyQ protein of *E. coli* was used for molecular docking studies (PDB ID: 7EIV_A).[27] Protein structures were prepared by removing water molecules, assigning bonding orders, adding the hydrogen atoms and Gasteiger–Marsili charges to the crystal structure. Finally, the prepared receptor structure was saved in PDBQT format to perform molecular docking.[28] For docking studies, a grid box was generated using AutoDock Vina into Center_X: 12.385 Å, Center_Y: 78.884 Å, and Center_Z: 168.705 Å grid points.

Selection and preparation of bioactive compounds. The current study used the StreptomeDB library which is known as the largest database of natural products from *Streptomyces* spp. and contains 6524 unique compounds.[29] Each compound in the library was prepared as follows: nonpolar hydrogen bonds were merged, Gasteiger–Marsili charges were added, atoms were matched to AutoDock atom types, and rotatable bonds. Energy minimization (MMFF94 force field) was assigned. Finally, the compounds were then converted to PDBQT format using Open Babel software.[28]

Virtual screening and molecular docking studies. In this work, a structure-based virtual screening approach was used to identify potential compounds to inhibit GlyQ protein. Therefore, 6524 secondary metabolites of *Streptomyces* spp. from the StreptomeDB library were docked with the selected aminoacylation cavity using AutoDock Vina. Docking results were then ranked based on binding affinity and clustered based on binning clustering (similarity cut-off = 0.5) and physicochemical properties (OpenBabel Descriptors) using ChemMine web tools. The Tanimoto coefficient is generally used as a similarity measure in ChemMine tools.[30]

ADMET evaluation and hit compounds selection. In this step, the selected compounds from the previous step were analyzed based on absorption, distribution, toxicity, metabolism, and excretion (ADMET) properties using the ADMETlab 2.0 database.[31] The compounds were selected based on Lipinski rule of five (RO5)[32] and introduced as final hit compounds. This rule is used to evaluate the orally active compounds based on 4 characteristics, including molecular weight < 500 Da, Xlogp (octanol–water partition coefficient) < 5, H-bond acceptor < 10, and H-bond donors < 5.[33]

Results

Upstream analysis

Pan/core-proteome analysis. The pipeline of this study (upstream analysis) is presented in Figure 1. The number of core proteins among 4 bacterial strains was 441. The core proteome accounted for 32% of the pan proteome, which was selected to identify common drug targets against these 4 bacterial strains.

Essential cytoplasmic proteins. Detailed analysis of the localization of determined proteins disclosed that out of 441 core proteins, a total of 99 non-cytoplasmic proteins (75 cytoplasmic membrane proteins, 17 unknown proteins, 5 periplasmic proteins, and 2 extracellular proteins) were excluded from this study. Overall, 342 cytoplasmic proteins remained. Among these cytoplasmic proteins, 210 essential proteins were recognized.

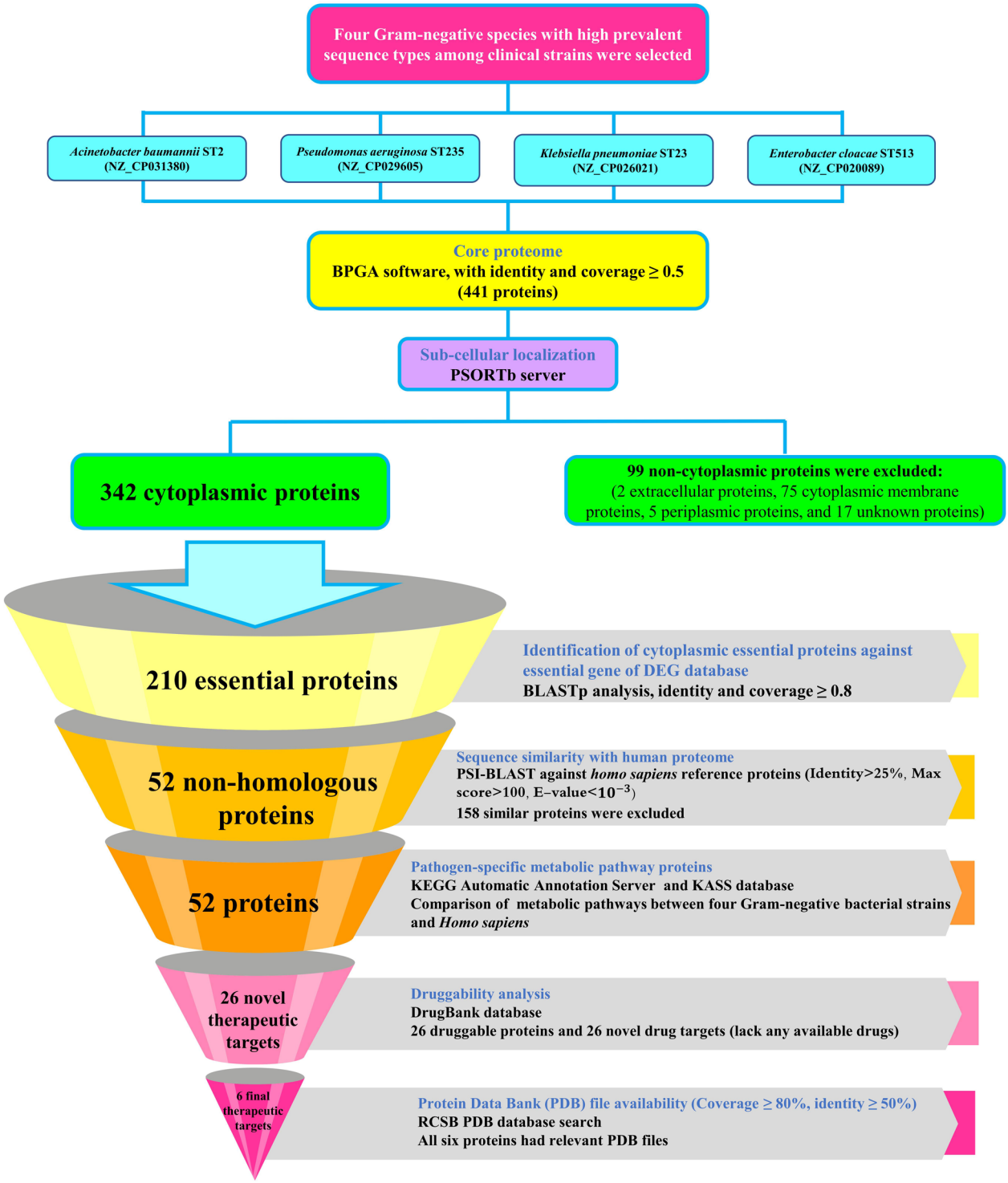


Figure 1. The pipeline of this study (upstream analysis). As illustrated, the number of the core proteome among 4 bacterial strains (*A. baumannii*, ST2; *P. aeruginosa*, ST235; *K. pneumoniae*, ST23; and *E. cloacae*, ST513) was 441. Using various bioinformatics servers and software, 6 common and novel drug targets were eventually identified. All targets were essential proteins with no similarity to the human proteome while having homologue or identical PDB files. KEGG indicates Kyoto Encyclopedia of Genes and Genomes; PDB, protein data bank; PSI-BLAST, Position-Specific Iterative Basic Local Alignment Search Tool.

The Similarity of drug targets against the human proteome. PSI-BLAST of essential proteins against human proteome revealed 52 non-homologous proteins. However, 158 proteins with high similarity to human proteome were excluded.

Pathogen-specific metabolome proteins. To identify unique pathways that exclusively belonged to the selected *Gram-negative* bacterial strains, metabolic pathways of non-homologous proteins also were investigated in the human and selected strains.

The BLASTp analysis demonstrated that all 52 proteins were absent in human metabolic pathways and exclusively related to the metabolome of these 4 strains. These proteins were involved in diverse vital metabolic functions. Most of them are associated with ribosome biogenesis (11 proteins, 21.1%), biosynthesis of the amino acid (7 proteins, 13.4%), and transcription and translation factors (7 proteins, 13.4%).

Druggable proteins and novel drug targets. Out of 52 metabolome-related proteins of these pathogens, 26 proteins resembled the targets of FDA-approved or experimental drugs in the DrugBank database. The remaining 26 proteins, with no similarity to the known drug targets, were considered novel and putative therapeutic targets. See Supplementary Data 1.

Sequence conservancy of novel drug targets among 4 Gram-negative bacteria. The Consurf analysis results disclosed that DapF, PR-S20, and GlyQ showed the highest conservation among mentioned bacterial strains. However, PR-L21, PR-L31, RpoN, and NusG showed the lowest conservancy. The essentiality parameter was determined based on the literature reviewing the effects of mutation on the physiology and survival of the microorganism, and 13 highly essential proteins were identified. See Table 1. Among 13 proteins, RP-L21, RP-L28, RP-L30, RP-L31, RP-S20, RP-S21, Rnt, and NusG had relevant PDB files with an identity $\geq$ 80%. The accession number of PDB files is mentioned in Table 1.

Protein–protein interaction network. The STRING analysis revealed that most of the putative drug targets, including RP-L21, RP-L28, RP-L30 (RpmD), RP-L31 (RpmE), RP-S20 (RpsT), and RP-S21 (RpsU), were ribosomal protein subunits and had protein–protein interactions to each other. The GlyQ protein had interactions with proteins involved in tRNA aminoacylation for protein translation, such as GlyS, PheS, HisS, and YsaB. The NusG, SoxR, and InfA interact with ribosomal proteins and transcription. The DapF interacted with proteins involved in peptidoglycan synthesis. The Rnt interacted with RNA ribonuclease proteins. Whereas, RpoN protein interaction network is not well-known. See Supplementary Data 2 and 3.

Shortlisted proteins. The therapeutic targets were assessed based on 4 characteristics, including relevant PDB file availability (coverage $\geq$ 80% and identity $\geq$ 50%), conservation among Gram-negative bacteria, and STRING analysis. Finally, 6 proteins were selected as novel therapeutic targets as follows: RP-L28, RP-L30, RP-S20, RP-S21, GlyQ, and Rnt. Due to the specific function of GlyQ as a unique factor in bacteria, this study concluded to select it for further analysis in the downstream section.

Downstream analysis

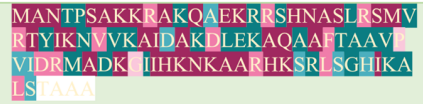
Homology modeling and multiple sequence alignment in 1D and 3D layers. Assessing the primary structure of the GlyQ protein revealed that the length of this protein is 325 aa in *A. baumannii*, 315 aa in *P. aeruginosa*, and 303 aa in *E. cloacae, K. pneumoniae*, and *E. coli*. The results are shown in Figure 2A. In addition, multiple sequence alignment in the 3D layer showed that their structure is highly identical (purple color). The non-overlapping amino acids are shown in gray color (Figure 2B). Furthermore, we selected the 3D structure of the GlyQ protein of *E. coli* (PDB ID: 7EIV_A) as a receptor. In addition, the 3D structural alignment of the GlyQ protein from *E. coli* and human glycyl-tRNA synthetase showed that they do not overlap the regions of GlyQ (Figure 2C).

Receptor structure and binding cavity. The aminoacylation cavity was included as an investigative position in the MOLE 2.5 software. Analysis of this software and literature review revealed that the amino acids Thr 44, Cys 45, Gln 60, Arg 64, Asp 67, Asn 75, Arg 76, Gln 82, Gln 84, Asp 119, Trp 121, Glu 122, Asn 123, Leu 126, Glu 141, Gln 144, Arg 169, and Tyr 245 are located in this cavity, therefore, it is suitable for grid box detection.

Virtual screenings and docking results. Docking results were ranked based on binding affinity. The threshold for binding affinity was set at −14 kcal/mol. Based on this threshold, 56 compounds with the lowest docking energy and the best conformation were selected as hit compounds. The binding affinity scores for these compounds were as follows: −14 kcal/mol (32 compounds), −15 kcal/mol (16 compounds), −16 kcal/mol (5 compounds), −17 kcal/mol (2 compounds), and −18 kcal/mol (1 compound). The predicted physicochemical properties of these 56 compounds were summarized in Supplementary Data 4. According to the obtained results, compounds with antitumor activity, cytotoxicity, and unknown function were excluded from the study. In the next step, of the remaining 33 compounds, 7 compounds with a logP above 5 were excluded from the study. Finally, the remaining compounds were categorized into 21 different clusters based on the similarity analysis by ChemMine tools.

ADMET evaluation and hit compounds selection. Finally, 5 compounds were selected as hit compounds according to Lipinski rules. Lipinski rules were developed to establish guidelines for "druggability" from the perspective of oral bioavailability for small molecules.[34] The final selected hit compounds were as follows: Rimocidin, Flavofungin, Chaxamycin, 11,11′-O-dimethyl-14′-deethyl-14′-methylelaiophylin, and Platensimycin (Figure 3). Among them, 11,11′-O-dimethyl-14′-deethyl-14′-methylelaiophylin had the highest human intestinal absorption. The interaction of 11,11′-O-dimethyl-14′-deethyl-14′-methylelaiophylin with the aminoacylation cavity of GlyQ protein is illustrated in Figure 4. The full results of this investigation are shown in Table 2. To access the ADMET properties of all 21 ligands, please refer to Supplementary Data 5.

Table 1. The relevant PDB files of novel and common therapeutic targets were identified in the BLASTp database, and conservation of the proteins among *A. baumannii*, *P. aeruginosa*, *K. pneumoniae*, *E. cloacae*, *and E. coli*, were evaluated using the ConSurf database.

Pathway	Gene name	Function	Accession number	Essentiality	PDB files availability				Conservation
					Description	Accession number	Coverage	Identity	The conservation scale: 1 2 3 4 5 6 7 8 9 Variable Average Conserved
Lysine biosynthesis	*dapF*	Diaminopimelate epimerase	WP_000923487.1	+++	Chain A, Diaminopimelate epimerase [*Acinetobacter baumannii* AB307-0294]	5HA4_A	99%	87.5%	
Ribosome biogenesis	*RP-L21*	Large subunit ribosomal protein L21	WP_003094761.1	+++	*Pseudomonas aeruginosa* 50s ribosome from a clinical isolate	6SPD_R	100 %	100%	
	RP-L28	Large subunit ribosomal protein L28	WP_003096556.1	+++	*Pseudomonas aeruginosa* 50s ribosome from a clinical isolate with a mutation in uL6	6SPB_X	98%	100%	
	RP-L30	Large subunit ribosomal protein L30	WP_001140434.1	+++	Select seq pdb|3J7Z|Z Structure of the *E. coli* 50S subunit with ErmCL nascent chain [*Escherichia coli* K-12]	3J7Z_Z	100 %	96.61%	
	RP-L31	Large subunit ribosomal protein L31	WP_001200845.1	+++	Chain W, 50S ribosomal protein L31 [*Acinetobacter baumannii* ATCC 19606 = CIP 70.34 = JCM 6841]	6YSI_W	100 %	100%	
	RP-S20	Small subunit ribosomal protein S20	WP_003094744.1	+++	*Pseudomonas aeruginosa* 70s ribosome from an aminoglycoside resistant clinical isolate [*Pseudomonas aeruginosa*]	6SPF_t	94%	100%	
	RP-S21	Small subunit ribosomal protein S21	WP_001144069.1	+++	Crystal structure of the bacterial ribosome from *Escherichia coli* at 3.5 A resolution. This file contains the 30S subunit of one 70S ribosome. The entire crystal structure contains two 70S ribosomes and is described in	2AVY_U	100 %	100%	

(Continued)

Table 1. (Continued)

					remark 400. [*Escherichia coli*]				
Aminoacyl-tRNA biosynthesis	*glyQ*	Glycyl-tRNA synthetase alpha chain	WP_000085212.1	+++	Chain A, Glycine--tRNA ligase alpha subunit [*Escherichia coli* K-12]	7EIV_A	89%	74.39%	
Sigma factors	*rpoN*	RNA polymerase sigma-54 factor	WP_003094357.1	+++	Chain M, RNA polymerase sigma-54 factor [*Klebsiella pneumoniae*]	5UI8_M	100 %	53.40%	
Transfer RNA biogenesis	*rnt*	Ribonuclease T	WP_003092070.1	+++	2.1 A crystal structure of *Pseudomonas aeruginosa* rnase T (Ribonuclease T) [*Pseudomonas aeruginosa* PAO1]	2F96_A	99%	97.31%	
Ribosome biogenesis	*nusG*	Transcription termination/antiterminator protein	WP_003862341.1	+++	Structure of THE RNA POLYMERASE LAMBDA-BASED ANTITERMINATION COMPLEX [*Escherichia coli* O157:H7]	6GOV_G	87%	93%	
Transcription factors	*soxR*	Redox-sensitive transcriptional activator	WP_003089301.1	+++	Crystal structure of SoxR in complex with DNA [*Escherichia coli* K-12]	2ZHG_A	90%	62.41%	
Translation factors	*infA*	Translation initiation factor IF-1	WP_001284370.1	+++	NMR structure of IF1 from *Pseudomonas aeruginosa* [*Pseudomonas aeruginosa*]	2N78_A	97%	77.46%	

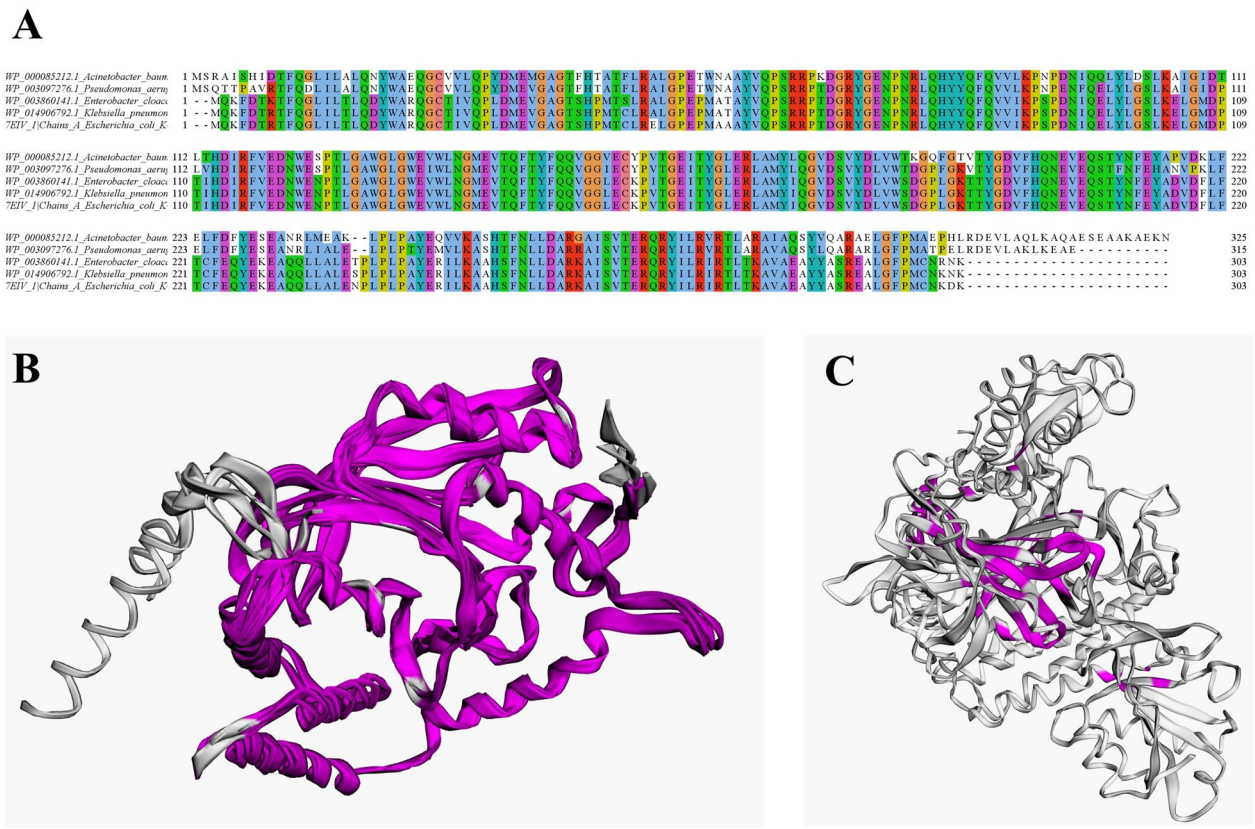


Figure 2. (A) Multiple sequence alignment in the 1D layer between the glycyl-tRNA synthetase (GlyQ) of *A. baumannii, P. aeruginosa, E. cloacae, K. pneumoniae*, and *E. coli.* (B) Multiple sequence alignment in the 3D layer between 4 predicted proteins and GlyQ protein from *E. coli*. (C) The 3D structural alignment of the GlyQ protein from *E. coli* and human glycyl-tRNA synthetase. The purple color indicates the overlap of the proteins, and the gray color shows the difference in 3D structure.

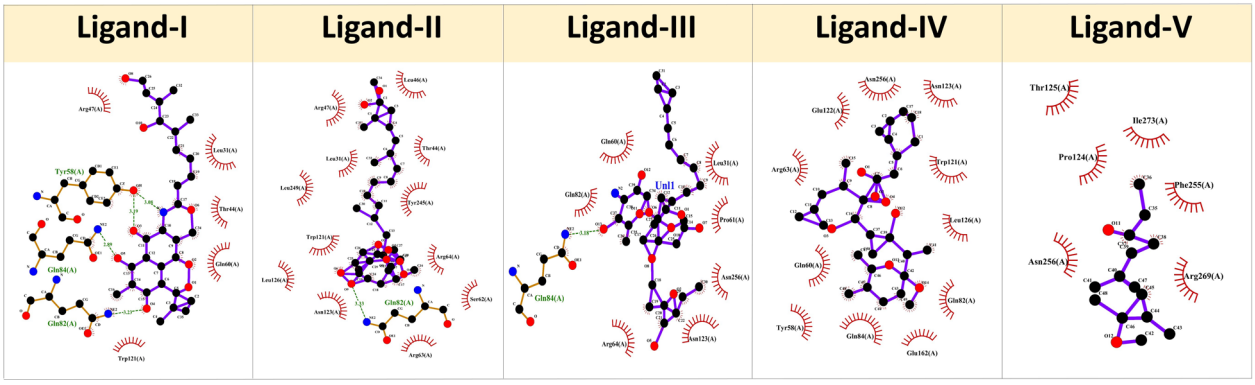


Figure 3. The interactions of selected hit compounds (I: Chaxamycin, II: Flavofungin, III: Rimocidin, IV: 11,11′-O-dimethyl-14′-deethyl-14′-methylelaiophylin, and V: Platensimycin) with involved amino acids in the cavity of glycyl-tRNA synthetase (GlyQ).

Discussion

Antimicrobial resistance is a multifactorial phenomenon. For example, a key factor in the emergence of resistance is the lack of proper antibiotic stewardship, leading to misuse of antibiotics, empirical treatment, and delays in accurate diagnosis and de-escalation of treatment. Over time, fewer and fewer effective antimicrobials are available against these infections, further escalating the problem. This problem is becoming increasingly baffling, especially in hospitals and intensive care settings.[35] Consequently, it continues to be a great need for new antimicrobial agents, especially those targeting multidrug-resistant Gram-negative bacteria.[36]

Advancing a new drug to market requires a significant investment in time and financial resources. Despite increased antimicrobial resistance, an indisputable need for new antimicrobial agents, and the development of new antibiotics is waning.[37] However, computational and bioinformatic approaches have accelerated drug development procedures.[38] During drug

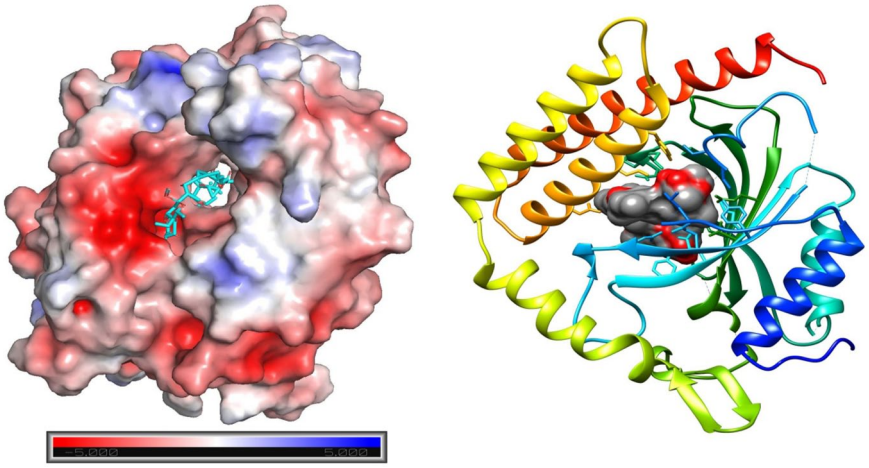


Figure 4. The interaction of 11,11′-O-dimethyl-14′-deethyl-14′-methylelaiophylin with aminoacylation cavity of glycyl-tRNA synthetase (GlyQ). The adaptive Poisson–Boltzmann Solver (APBS) electrostatics has been presented on protein (right hand). The cartoon structure of protein and involved amino acids with ligands have been shown (left hand).

Table 2. Selection of 5 hit compounds with druggability and oral bioavailability based on Lipinski rules.

ADMET COMPOUND	HIA[a]	PPB[b] (%)	BBB[c] PENETRATION	METABOLISM	EXCRETION (CL ML/MIN/KG)	TOXICITY	PAINS[d]	DOCKING ENERGY (KCAL/MOL)
Rimocidin	+	87.727	—	CYP2C19 substrate CYP2D6 substrate CYP3A4 substrate	−0.142	+++	0	−15
11,11′-O-dimethyl-14′-deethyl-14′-methylelaiophylin	+++	98.652	—	CYP2C19 substrate CYP2D6 substrate CYP3A4 substrate	1.041	+++	0	−15
Platensimycin	–	99.609	—	CYP2C19 substrate CYP2C9 substrate CYP3A4 inhibitor CYP3A4 substrate	0.954	+++	0	−15
Chaxamycin	–	98.646	—	CYP2C19 substrate CYP2C9 inhibitor CYP3A4 inhibitor CYP3A4 substrate	0.829	+++	0	−14
Flavofungin	-	82.051	—	CYP1A2 substrate CYP2C19 substrate CYP3A4 substrate	−0.114	+++	0	−14

Abbreviation: ADMET, absorption, distribution, toxicity, metabolism, and excretion.
[a]Human intestinal absorption.
[b]Plasma protein binding.
[c]Blood–brain barrier.
[d]Pan-assay interference compounds.

development, the critical bioactivities of drug candidates should be investigated, including their efficacy, pharmacokinetics, and adverse effects. Advances in chemical synthesis and biological screening techniques during the last decade have generated a large amount of data for millions of small molecules stored in different databases. These data, along with novel machine learning approaches, provided a great opportunity to facilitate drug discovery and development by predicting in vitro, in vivo, and clinical outcomes. Computer-aided drug discovery (CADD) is an essential approach in modern preclinical drug discovery. This approach combines various computational techniques and software programs to achieve the desired outcome.[39,40]

In the current study, we attempted to select common drug targets against 4 superbugs considering several criteria, such as localization in cytoplasm 6 cytoplasmic, having no role in human metabolic pathways, and no cross-reactivity with the human proteome. Thus, among the 441 core proteins of these species, only 6 proteins met our considered criteria that most of them were involved in ribosome biogenesis, biosynthesis of the

amino acid, transcription, and translation. Ribosomal and translational machinery proteins are potential targets for antibiotic discovery in bacteria.[41] For example, erythromycin, chloramphenicol, linezolid, and tetracycline are antibiotics that inhibit the synthesis of proteins in bacterial cells. Mechanisms of inhibition action include interrupting peptide chain elongation, blocking the A site of ribosomes, misreading the genetic code, or preventing oligosaccharide side chains from binding to glycoproteins.[42,43]

The 70S ribosomes of bacteria and 55S ribosomes of human mitochondria have similar structures, physiology, and mechanism of action. Moreover, the structure of pathogenic and non-pathogenic bacteria is almost similar. Thus, antibiotics-targeted ribosomal proteins would have a serious cross-reaction with human mitochondrial ribosomes and inhibit both pathogenic and non-pathogenic bacteria. Moreover, there are some major challenges in the development of new ribosome-targeting antibiotics, including the structural complexity of 70S ribosomes, extensive resistance mechanisms, and limited in vitro transcription/translation assay systems to assess the function of ribosomal inhibitors. NMR spectroscopy is not able to analyze the structure, intermolecular interactions, and dynamic changes of supermolecules, such as ribosomal proteins which have high molecular weight. Similarly, the X-ray diffraction technology is not suitable for ribosomal proteins due to the difficult crystallization of ribosomes. Considering the complex structure of bacterial ribosomes, targeting the pharmacophore of previous ribosomal antibiotics using ligand-based drug discovery might be a more appropriate alternative to receptor-based docking studies and molecular dynamics (MD).[44]

Taken together, we put aside the ribosomal proteins (e.g., RP-L28, RP-L30, RP-S20, and RP-S21) and GlyQ were selected to find suitable ligands in the downstream analysis using the StreptomeDB library. The StreptomeDB library is an extended resource of natural products produced by *Streptomyces* spp.[45] Natural products (NPs) have indispensable roles in drug discovery, especially against infectious diseases and cancer. The NPs have been structurally "optimized" by evolution to perform specific biological functions, including regulating endogenous defense mechanisms and interacting with other organisms, which explains their great importance in infectious diseases and cancer. In addition, their use in traditional medicine may shed light on their efficacy and safety. Overall, the pool of NPs is enriched with "bioactive" compounds that cover a wider range of chemical space than typical synthetic libraries of small molecules.[46,47]

There are 2 types of glycyl-tRNA synthetase (GlyRS) in different organisms. Eukaryotes, archaea, and some bacteria own a homodimeric GlyRS that belongs to the typical class II aaRSs. However, other bacteria have a heterotetrameric GlyRS with 2 α-subunits and 2 β-subunits to produce glycyl-tRNA^{Gly}. The α-subunits encode the central catalytic domain of GlyRS with all 3 characteristic motifs of the II aaRSs class; however, the sequence of the larger subunit (β) is different from α2 GlyRS or other II aaRSs. In bacteria, GlyQ protein (αβ) 2 belongs to the class II aminoacyl-tRNA synthetase family and has a role in protein translation. This gene is a member of the T-box regulon and catalyzed the following reaction: ATP + glycine + tRNA ^{Gly} –> AMP + diphosphate + glycyl-tRNA ^{Gly}.[48] The crystal structure of *E. coli* GlyRS demonstrated that the β-subunits consist of 5 domains. β-subunit domains might perform 2 different functions, forming the active pocket along with α-subunit, and binding to tRNA due to the similar structure to tRNA CCA-adding enzymes and a tRNA recognition domain in alanyl-tRNA synthetase (AlaRS).[49] The α-subunit alone has no or very low activity even for amino acid activation, suggesting that both the α- and β-subunits are essential for the aminoacylation of tRNA^{Gly}.[50] From a practical perspective, its unique properties provide greater chemical space for the search for bacteria-specific inhibitors that do not cross-react with human GlyRS. This approach enables us to explore non-toxic antibiotics.[49] The ADMET evaluation of 21 compounds showed that only 5 of them can be accepted according to Lipinski RO5. This rule is used to determine whether a chemical compound with a particular pharmacological or biological activity has chemical and physical characteristics to be used as an orally active drug for humans.[51] According to RO5, an orally active drug with up to 5 hydrogen bond donors, and at most 10 hydrogen bond acceptors, a molecular weight < 500 Daltons, and an octanol–water partition coefficient (logP) should not exceed 5.[52]

In our study, virtual screening led to the identification of 5 hit compounds against GlyQ, including Rimocidin, Flavofungin, Chaxamycin, 11,11′-O-dimethyl-14′-deethyl-14′-methylelaiophylin, and Platensimycin. It was reported that *Streptomyces leeuwenhoekii* isolated from the hyperarid Atacama Desert produces the ansamycin-like compounds named Chaxamycins, which have potent antibacterial activity and moderate antiproliferative activity.[53] *S. leeuwenhoekii* produces 4 compounds. including Chaxamycin A, Chaxamycin B, Chaxamycin C, and Chaxamycin D. Among them, Chaxamycin D showed highly selective activity against *Staphylococcus aureus* ATCC 25923 and methicillin-resistant *S aureus* (MRSA) strains, while Chaxamycin A acts as an inhibitor of the intrinsic ATPase activity of Hsp90 in human, which is involved in cancer proliferation.[54]

In addition, Flavofungin, a polyene macrolide antibiotic, was presented in this study as a hit compound with acceptable binding affinity to the aminoacylation cavity of GlyQ protein. Polyene macrolides are important therapeutic agents for the treatment of fungal infections. Their structure consists of 4 to 8 conjugated carbon–carbon double bonds with an e-configuration. This structural unit leads to the rod-like shape of these molecules. This conformation probably plays an important role in binding to sterol molecules of fungi, leading to the antifungal activity of these antibiotics.[55] The results of this

study indicate that this antibiotic may be effective against 4 Gram-negative bacteria. The use of such compounds may be useful, especially in the case of co-infection of fungi and bacteria.

Rimocidin is a 28-membered tetraene macrolide with a large lactone ring and a sugar component. It mainly targets the fungal cell membrane and leads to the destruction of electrochemical gradients, loss of ions, and eventual cell death via ergosterol-forming channels. Rimocidin has recently attracted the attention of researchers due to its wide-spectrum bioactivity against a variety of pathogenic microorganisms and its potential as a fungicide.[56]

In 2006, Platensimycin was isolated from *Streptomyces platensis* in a soil sample in South Africa. This compound is a useful antimicrobial agent, due to the bacteriostatic effects and lack of any cross-resistance to other classes of antibiotic-resistant bacteria. Moreover, it is relatively non-toxic to humans and only targets bacterial fatty acid synthesis. This antibiotic is effective against antibiotic-resistant Gram-positive pathogens and *Mycobacterium tuberculosis*.[57]

Streptomyces spp. 7 to 145 were confirmed to have the potential to produce glycosidic antibiotics. Chemical analysis of culture extracts of this strain revealed 2 new 6-deoxyhexose-containing antibiotics, 11′, 12′-dehydroelaiophyline, and 11,11′-O-dimethyl-14′-deethyl-14′-methylelaiophyline. These compounds showed good antibacterial activity against methicillin-resistant *S. aureus* and vancomycin-resistant *Enterococci*.[58] The results of this study revealed that 11,11′-O-dimethyl-14′-deethyl-14′-methylelaiophylin may inhibit GlyQ protein in Gram-negative bacteria. Moreover, this compound showed high absorption capacity in the human intestine. Absorption refers to the process by which an orally administered drug is absorbed into the bloodstream by the gastrointestinal system.[59] Despite the promising results of this study, we should mention that this investigation had some limitations. For example, the obtained results are based on bioinformatics methods, therefore, further in vitro and in vivo studies are needed to investigate the bactericidal effects of proposed therapeutic agents against these microorganisms. Moreover, the main target of this study was presenting new targets and inhibitors against mentioned bacteria and MD simulation will be performed in the future of these investigations.

Conclusions

Infections caused by multidrug-resistant Gram-negative bacteria have increased worldwide and efforts must be made to develop new antibiotics that can successfully cure Gram-negative superbugs. The results of this study provide 6 common putative drug targets against highly resistant Gram-negative bacteria (e.g., *A. baumannii, K. pneumoniae, P. aeruginosa*, and *E. cloacae*) including RP-L28, RP-L30, RP-S20, RP-S21, GlyQ, and Rnt. Moreover, we proposed 5 different hit compounds (e.g., Rimocidin, Flavofungin, Chaxamycin, 11,11′-O-dimethyl-14′-deethyl-14′-methylelaiophylin, and Platensimycin) against GlyQ protein as a novel therapeutic target. Altogether, 11,11′-O-dimethyl-14′-deethyl-14′-methylelaiophylin has been identified as a promising agent with potentially inhibiting effects on GlyQ protein in Gram-negative bacteria with a high absorption capacity in the human intestine. Research on broad-spectrum antibiotics that are effective against Gram-negative superbugs is important to combat nosocomial infections. The results of this study can form the basis for discovering broad-spectrum therapeutics for highly resistant Gram-negative bacteria. However, further in vitro and in vivo studies are needed to investigate the bactericidal effects of proposed therapeutic agents against these microorganisms.

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Author Contributions

S.F. wrote the original draft, validated the data, and had a role in investigation and conceptualization. N.N.G. wrote the original draft and made critical revisions. H.K. had a role in data curation. H.R. had a role in the investigation. S.M.B. reviewed the analysis. F.B. had a critical role in the conceptualization, methodology, project administration, supervision, validation, and writing. All authors reviewed and approved the final manuscript.

Ethics Approval

This article does not contain any studies with humans or animals performed by any of the authors.

Supplemental Material

Supplemental material for this article is available online.

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