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Determining the 3-substituted Coumarins inhibitory potential against the HslV protease of *E. coli*

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Abstract. – OBJECTIVE: The growing bacterial resistance towards classical antibiotics demands the development of novel approaches for the effective treatment of potentially fatal bacterial infections in humans. Proteostasis is crucial for the survival of every living cell, as several important physiological functions depend on well-regulated proteostasis. Within bacteria, the regulation of proteostasis relies on AAA+ (Adenosine 5'-triphosphatases associated with diverse cellular activities), ATPases, such as the HslVU complex (heat shock locus gene products U and V), along with other proteases. The HslVU protease/chaperon complex is thought to be the progenitor of the eukaryotic proteasome that regulates proteostasis mostly in prokaryotes. This study aimed to determine the inhibitory potential of 3-substituted coumarin derivatives against *Escherichia coli* heat shock locus V (HslV) protease.

MATERIALS AND METHODS: In this study, twenty-three derivatives of 3-substituted coumarin were assessed for their inhibitory potential against *E. coli* HslV protease using both *in-vitro* and *in-silico* techniques.

RESULTS: Among all the tested compounds, US-I-64, US-I-66, US-I-67, and US-I-68 displayed notable inhibitory potential against the HslV protease, showing IC_{50} (half maximal inhibitory concentration) values ranging from 0.2 to 0.73 μM. Additionally, the inhibitory potential of these compounds against the eukaryotic proteasome was also evaluated using a separate *in-silico* study. It was found that these compounds did not bind with the proteasomal active site, suggesting no apparent side effects of these lead molecules.

CONCLUSIONS: These identified HslV protease inhibitors can be used for the development of novel and safer anti-bacterial drugs.

Key Words:
Coumarins, Inhibitory potential, HslV protein, ATPases, *E. coli*.

Introduction

Antibiotic resistance poses one of the greatest threats to modern drug discovery. It is becoming increasingly prevalent and has rendered a significant number of antibacterial drugs ineffective, which poses a major concern in the treatment of bacterial infectious diseases[1,2]. This alarmingly increasing antibiotic resistance toward existing drugs demands the development of novel strategies to treat bacterial infections.

Proteostasis involves the degradation of non-native or misfolded proteins inside the cells, which is considered an indispensable phenomenon for cell survival. Several ATP-dependent proteases, notably the HslVU protease/chaperone complex, are responsible for the maintenance of proteostasis in bacteria. The HslVU protease/chaperon complex comprises two distinct subunits, i.e., the heat shock locus U (HslU) ATPase, which unfolds and translocates the aberrant protein substrates, and the heat shock locus V (HslV) protease, which degrades those proteins[3]. The overall architecture of the HslVU protease/chaperon complex and eukaryotic proteasome is quite similar. Two hexamers of HslV protomers join to form the central proteolytic core of the complex. While the hexamers of HslU ATPase bind to either side of the HslV dodecamer in the presence

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of ATP to form the HslVU protease/chaperon complex. HslV protease is considered a homolog of 20S proteasome and carries an N-terminal threonine active site. Both the 26S proteasome and the HslVU protease complex share close structural similarities and belong to the same N-terminal nucleophile (Ntn) hydrolase family. Based on this structural and biochemical analogy, the HslV protease is considered the progenitor of the eukaryotic proteasome and is often referred to as miniproteasome[4,5]. Notably, the classic proteasome inhibitors Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG-132), lactacystin, and 4-iodo-3-nitrophenyl Acetyl-leucinyl-leucinyl-leucinyl-vinylsulfone (NLVS) have also been found to inhibit the HslV protease activity[6].

The HslV protease is crucial for the survival of bacterial cells as it regulates protein homeostasis and maintains the healthy cellular proteome within the cell with other ATP-dependent proteases[7,8]. The HslV protease is recognized for its ability to degrade many short-lived regulatory proteins, including *SulA*, s_{32}, Arc repressor, and RcsA[7,9,10]. When the hslVU-deleted mutants, both with and without concurrent deletion of other ATP-dependent proteases, including caseinolytic protease proteolytic subunit (ClpP), Lon, and Filamentous temperature sensitive H (FtsH), were analyzed, it was proved that the HslVU complex along with the other ATP-dependent proteases critically controls the heat shock response by regulating the degradation of σ^{32} and the existing misfolded and abnormal proteins in bacteria. Substantially, the deletion of HslVU leads to a temperature-sensitive phenotype[8,11]. Moreover, the HslV protease is crucial in promoting bacterial survival and its attachment with the host[12-14]. That is why, the HslV protease is considered a promising drug target in antibacterial drug discovery and its inhibition can be manipulated to develop new and efficient antibacterial drugs. The inhibition of bacterial proteases remains a successful strategy in antibacterial drug discovery and many studies[15-17] have been reported in this context. Recently, our group published some potential HslV protease inhibitors from different chemical scaffolds[18]. This research article is based on our latest work on coumarins, which are considered significant candidates in the process of drug discovery.

Coumarins are known for various biological activities including antibacterial, antioxidant, antiviral, and an-ticancer properties and relatively low toxicity[19,20]. These findings highlight the potential of different couma-rin derivatives in the field of medicine[21-25]. Furthermore, their inhibitory potential has also been reported against many enzymes, including acetylcholinesterase, carbonic anhydrase, and tyrosinase[26-28]. Even Psoralens or furanocoumarins have been recognized as potential inhibitors of the proteasome[29]. Specifically, 3-substituted coumarins having alkyl, aryl, benzyl sulfoxide, ferrocenyl, and trifluoroacetyl substitutions have been identified to possess effective anticancer, anti-inflammatory, and antibacterial properties[30-32].

In this study, the inhibitory potential of a different series of 3-substituted derivatives of coumarins was evaluated against both the HslV protease and the proteasome. Notably, these compounds are already known to exhibit antibacterial activities against different strains of multi-drug resistant *S. aureus*, including Epidemic methicillin-resistant *Staphylococcus aureus*[16] (EMRSA-16), EMRSA-17, methicillin-resistant *Staphylococcus aureus* (MRSA-252), and other local clinical isolates[33]. However, the specific mode of action of these compounds is still not known. In the present study, molecular docking, and *in-vitro* HslV inhi-bition studies revealed a possible molecular basis of the antibacterial activity of 3-substituted coumarins.

Materials and Methods

Homology Modeling

To obtain the active *E. coli* HslV conformation for ligand docking studies, homology modeling using MODELLER V9.15[34] was performed. The crystal structure of *Haemophilus influenzae* HslVU complexed with proteasomal inhibitor; NLVS with Protein Data Bank (PDB) ID "1KYI"[35] was used as the template. The stereochemistry and energy profile of the model was evaluated using PROCHECK (version 3.0)[36] and PROSA (version 4.0)[37] standalone software, respectively.

Molecular Docking Studies

Twenty-three derivatives of 3-substituted coumarin were sketched and optimized using Marvin Sketch[38]. The synthesis of these compounds has been reported previously[33]. The spectral data of compound synthesis has been given in **Supplementary Data**. AutoDock tools[39] were used to prepare the PDBQT files for molecular docking studies using AutoDock Vina (1.1.2) software[40]. The programs LigPlot+ (version 2.2.4)[41] and DS-Visualizer (version 16.1.0.15350)[42] were used

to analyze the results of molecular docking studies. The utilization of these software tools assists in the comprehensive analysis and interpretation of the molecular docking results, aiding in the selection and optimization of potential drug candidates. The compounds that exhibited the best docking scores were chosen for further analysis in an *in-vitro* assay. Additionally, the four lead molecules were docked into the binding pocket of the human proteasome (PDB ID: 5LF3)[43].

Molecular Dynamics (MD) Simulation

All four conformers consisting of the proteasome and the lead compounds obtained by molecular docking studies were subjected to MD simulations using Desmond, which is a package of Schrödinger LLC[44] version Schrödinger Release 2019-4. The MD simulations were carried out to get insights into protein-ligand interactions with the proteasome following molecular docking studies. The simulations were carried out for 100 ns for each complex in triplicates and root-mean-square-deviation (RMSD) values, root-mean-square-fluctuations (RMSF) values, and the protein-ligand interactions were calculated from trajectories. The protein-ligand complexes were first processed using the Protein Preparation Wizard tool of Schrödinger Maestro[45]. This preparation also included optimization and minimization of complexes. The Optimized Potentials for Liquid Simulations (OPLS_2005) force field was applied, and all the complexes were solvated in an orthorhombic box. A water model with the addition of 0.15 M (NaCl) of sodium and chloride ions to neutralize the charge was employed for system building. For MD-Simulation studies, an NTP ensemble with a temperature of 300 K and 1 atm pressure was chosen for the whole course of 100 ns MD simulation. Before running the simulations, the complexes were relaxed, and trajectories were saved every 10 ps.

The In-Vitro HslV Protease Inhibition Assay

The HslV protease and the HslU ATPase were expressed and purified as previously stated[46]. Top scoring derivatives of 3-substituted coumarin, i.e., US-I-64, US-I-66, US-I-67, and US-I-68 were synthesized and tested for their *in-vitro* inhibitory potential at various concentrations, including 0.1 µM, 0.25 µM, 0.5 µM, 1.0 µM, and 1.5 µM. The protease activity of HslV was evaluated using the reaction mixture containing HslV (5 nM), HslU (10 nM), Tris-HCl buffer (pH 8) containing 5 mM $MgCl_2$, 0.5 mM EDTA, 2 mM ATP, 0.1 mM Z-Gly-Gly-Leu-AMC (Sigma Aldrich, Burlington, MA, USA) along with 5% Dimethyl sulfoxide (DMSO), and the corresponding compound, as previously described[47]. NLVS, which is a proteasome inhibitor, was used at the concentration of 20 µM as the standard inhibitor. The inhibition of HslV protease was evaluated using a fluorescence-based assay. The measurement of fluorescence was conducted at 37°C by Varioskan LUX microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). The assay involved the use of a fluorogenic peptide substrate, Z-GGL-AMC, which releases 7-amino-4-methylcoumarin (AMC) upon protease activity. The fluorescence (λ_{ex}= 355nm, λ_{em}= 460 nm) emitted by the released AMC was measured, and the intensity of the fluorescence signal correlated with the enzymatic activity of the *E. coli* HslV protease. Percent inhibition of HslV protease was calculated using the following equation: % Inhibition = 100 x [1-(X-min) / (max-min)]. The IC_{50} values were calculated from the graph plotted *vs.* compound concentration and percent inhibition. Standard deviation (SD) and Standard error of mean (SEM) were calculated using MS Office Excel 365.

Results

Homology Modeling of E. coli HslV Protease

The three-dimensional structure of *E. coli* HslV protease in the active conformation was required to carry out molecular docking studies. Pair-wise sequence alignment revealed 80.23% identities, 98% query cover-age, and 0% gap between the two sequences. The overall quality of the HslV model was excellent concern-ing ProSa[37] and Procheck[36] parameters. The analysis of the Ramachandran plot of the model revealed that a significant majority of the residues i.e., 88.88%, were present in the most favored region of the plot. Only one amino acid residue was observed in the disallowed region, but it was not associated with any regular secondary structure but rather identified as part of a distant loop region that is not proximal to the active site. The Z-score of both the model and the template was found to be -8.29, suggesting an overall good quality of the homology model (Figure 1). Superimposition of the C-alpha backbones of the homology model of *E. coli* HslV protease and the crystal structure of *H. influenzae* HslV protease showed a lower RMSD value, i.e., 0.2 Å suggesting overall similar architectures (**Supplementary Figure 1**).

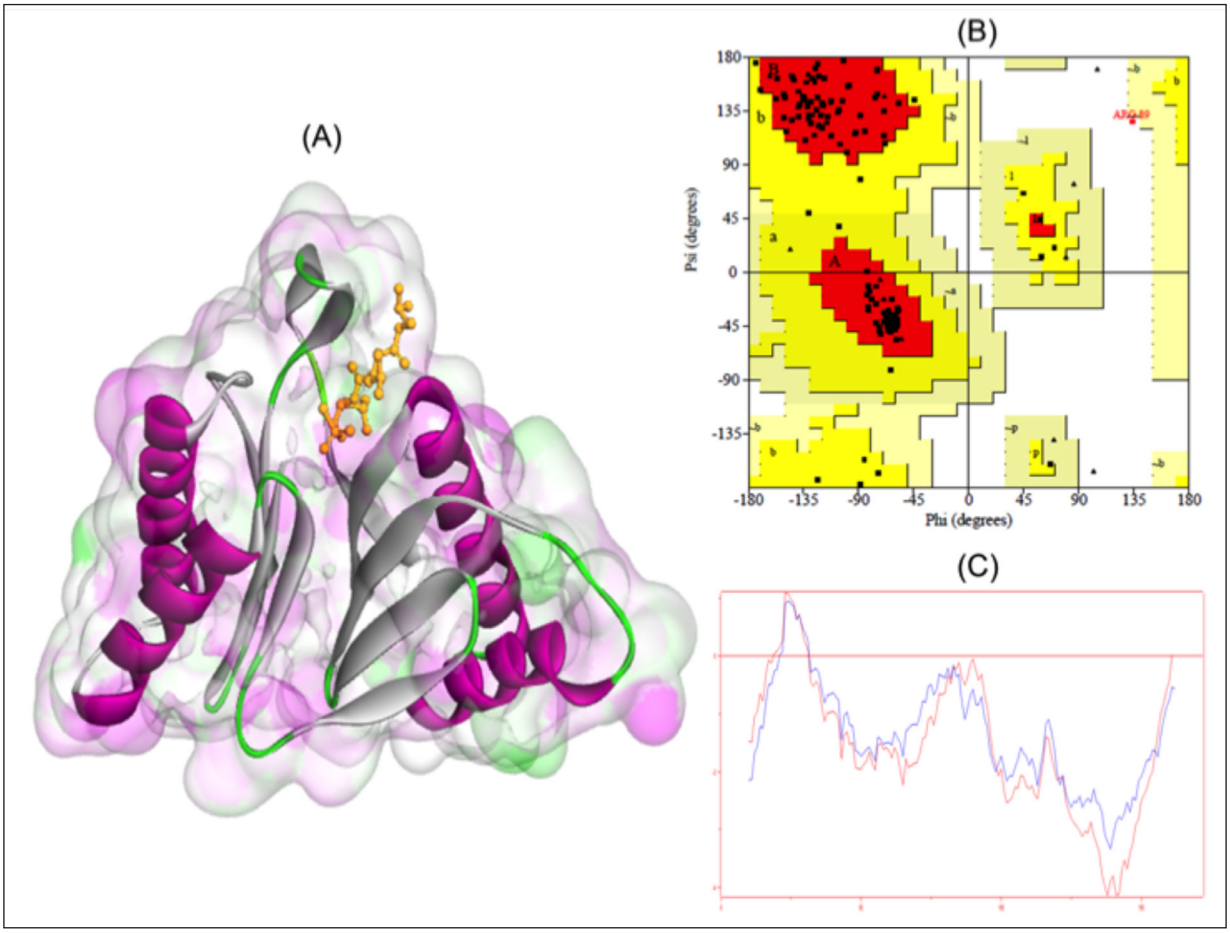

Figure 1. Homology model of *E. coli* HslV protease and its evaluation. **A**, Three-dimensional structure of *E. coli* HslV protease generated through homology modeling. The NLVS bound in the active site is shown by the orange color in the balls and sticks representation. **B**, The Ramachandran plot. **C**, The energy profile of the model.

Molecular Docking Studies of 3-Substituted Coumarins

Structure-based drug design typically involves the utilization of molecular docking, which is widely recognized as one of the most employed methods in this field and has been applied to identify potential drug candidates against various pathogens[48-51]. To find potential drug candidates that can effectively inhibit the *E. coli* HslV protease, virtual screening of twenty-three 3-substituted coumarin derivatives was performed. A known proteasomal inhibitor, NLVS, was used as the reference molecule[35].

In this study, a series of twenty-three compounds belonging to the 3-substituted coumarin class were checked for HslV protease inhibition. We conducted *in-silico*, as well as *in-vitro* investigations, to evaluate their potential as inhibitors. This library of compounds encompasses various analogues of coumarin, including 3-benzoyl, 3-acetyl, and 3-ethyl ester derivatives. When the proteasomal inhibitor NLVS was docked into the active site of HslV protease, a docking score of -6.3 kcal/mol (kilocalorie/mole) was obtained. Furthermore, when NLVS was re-docked into the active site of the HslV protease, the protein-ligand interactions were like those observed in the crystallized structure (Figure 2A). Four compounds including US-I-64, US-I-66, US-I-67, and US-I-68 showed comparatively higher docking scores (i.e., -8.4 kcal/mol, -8.5 kcal/mol, -8.6 kcal/mol, and -8.5 kcal/mol, respectively) than other compounds including the reference compound NLVS. The docking scores of all the docked compounds have been tabulated in **Supplementary Table I**. Out of all the compounds tested, the 3-benzoyl derivatives exhibited better docking scores compared with the acetyl and ethyl ester derivatives.

Molecular docking analysis revealed that the compounds with chloro, fluoro, and bromo

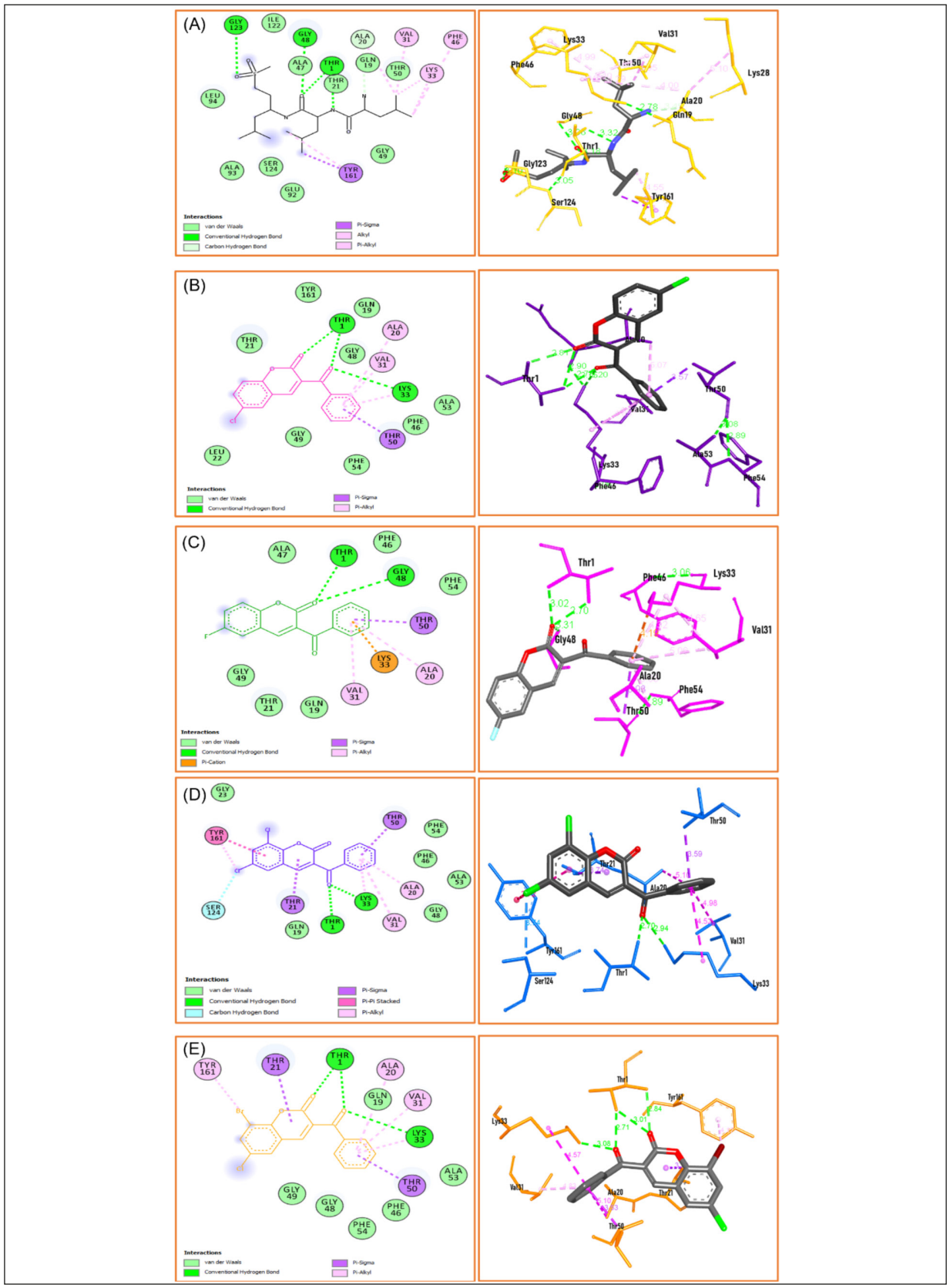

Figure 2. The two and three-dimensional interactions of the compounds docked into the active site of the *E. coli* HslV protease. (**A**), NLVS, (**B**), US-I-64, (**C**), US-I-66, (**D**), US-I-67, (**E**), US-I-68.

substitutions exhibited higher inhibitory potential against the HslV protease compared to other substitutions. Receptor-ligand interaction was predominantly established via hydrogen bonds between the ligands and the main catalytic residue, Thr1 (Figure 2). These hydrogen bonds facilitated the binding of the ligands into the active site of the HslV protease. Notably, the N-terminal threonyl Oγ nucleophile, connected with a covalent bond with NLVS in the template crystal structure, formed conventional hydrogen bonds with the best-docked ligands. Lys33, another important residue, was also involved in multiple bonds, including Pi-cation, Pi-alkyl, and conventional hydrogen bonds with the ligands. Pi-sigma interactions were also formed with Thr50 and Thr21. Pi interactions are crucial in protein-ligand interactions as they facilitate charge transfer between aromatic systems and, hence, play a significant role in stabilizing the protein-ligand complex and influencing the binding af-finity of the ligand to the protein[52]. The protein-ligand complex was additionally strengthened by van der Waals interactions with other important residues, including Gln19, Gly23, Phe46, Gly48, Ala53, and Phe54. The binding mode of these ligands with HslV was similar to that of NLVS (Figure 2A).

Two compounds, i.e., US-I-66 and US-I-67, made potential hydrogen bonds with the active site residue Thr1 showing a bond length of 2.70 Å. All these top four docked compounds have stronger hydrogen bonds than those between NLVS and HslV (3.18 Å). Notably, among all the four top-scoring compounds, the compound US-I-67 was found to be involved in making a potential hydrogen bond with another catalytic amino acid residue, Lys33, suggesting its stronger and more stable binding with the HslV protease.

The compound US-I-64 formed two hydrogen bonds with the N-terminal threonine and one hydrogen bond with Lys33 (Figure 2B). The compound US-I-66 also made a hydrogen bond with catalytic Thr1 and a Pi-cation bond with Lys33 (Figure 2C). The benzoyl derivatives, including US-I-67 and US-I-68, have two halogens attached at positions 6 and 8. The compound US-I-67 with two chlorine atoms showed an unusual carbon-hydrogen bond between chloride and Ser124. Tyr161 also made Pi-alkyl interaction with the same chloride atom (Figure 2D). This could be the reason for tighter binding and lesser IC_{50} value. Likewise, the compound US-I-68 with one chloride and one bromide also showed additional interaction. The bromide atom was also shown to interact with Tyr161 (Figure 2E). The remaining benzoyl derivatives with only one halogen atom seemed to bind slightly less efficiently as they did not interact with Tyr161 residue.

The chymotrypsin-like activity of proteasome was targeted for inhibition since it is important and most of the reported proteasome inhibitors act by mainly inhibiting this activity[53]. These four identified inhibitors of the HslVU protease did not show good interactions with the proteasomal active site as were observed with the HslV protease. The comparison of the docked poses of compounds into the active site of the HslV protease with that of the proteasome is shown in Figure 3. All four compounds

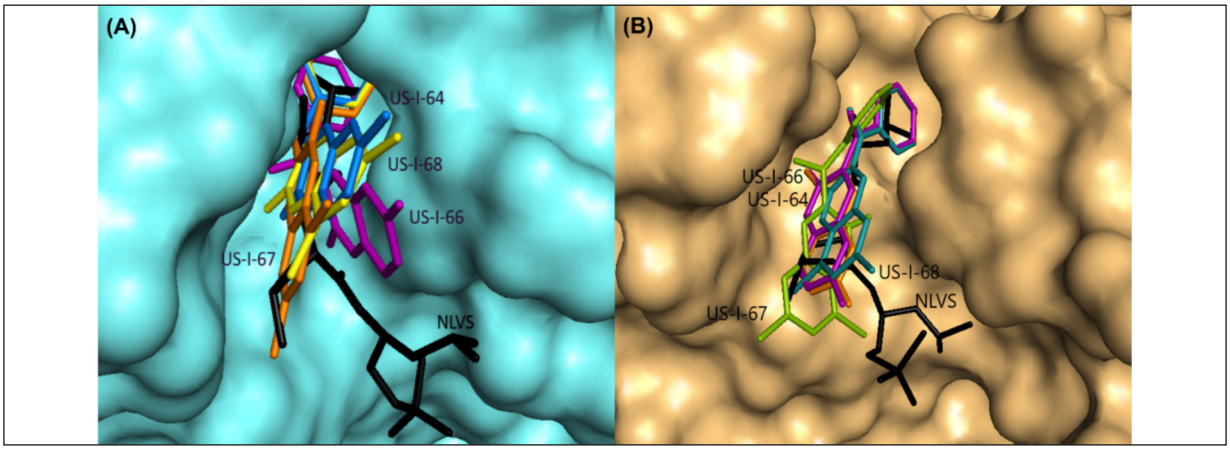

Figure 3. The three-dimensional poses of the top-scoring docked compounds. **A**, The active site of *E. coli* HslV protease shown by cyan color. **B**, The active site of proteasome shown by peach color. The compounds are shown in sticks representation by different colors.

showing the highest docking scores against the HslV protease docked less efficiently into the active site of the proteasome (Table I).

The compound US-I-64 did not bind into the active site of the proteasome but rather at another site and did not show any interactions with the active site residue threonine (Figure 4). The catalytic N-terminal threonine of the proteasome β subunit was observed to form conventional hydrogen bonds with only one ligand i.e., US-I-66. The other catalytically important residues, including Thr21, Lys33, Gly47, and Ser130, also stabilized the protein-ligand interactions by forming multiple bonds (Figure 5). The compound US-I-67 also bound into the active site of the proteasome but did not make strong interactions. Only a weaker and unconventional carbon-hydrogen bond was observed with the catalytic threonine. Moreover, the important residue Lys33 did not show any interaction with the ligand (Figure 6). The compound US-I-68 was distant from the active site and, therefore, did not show any interaction with the main catalytic residues (Figure 7).

Table I. Molecular docking scores represented in kilocalorie/mole (Kcal/mol) and half maximal inhibitory concentration (IC_{50}) values of the compounds with topmost docking scores. SEM denotes standard error of mean. NLVS is the abbreviation of 4-iodo-3-nitrophenyl acetyl-leucinyl-leucinyl-leucinyl-vinylsulfone).

Compound	Molecular weight	Docking Score with Proteasome (Kcal/mol)	Docking Score with HslV (Kcal/mol)	IC_{50} ± SEM (μM)
NLVS	722.636	-6.0	-6.3	11 ± 0.056
US-I-64	284.694	-7.6	-8.4	0.73 ± 0.029
US-I-66	268.239	-7.8	-8.5	0.62 ± 0.011
US-I-67	319.139	-7.6	-8.6	0.27 ± 0.01
US-I-68	363.590	-7.5	-8.5	0.44 ±0.026

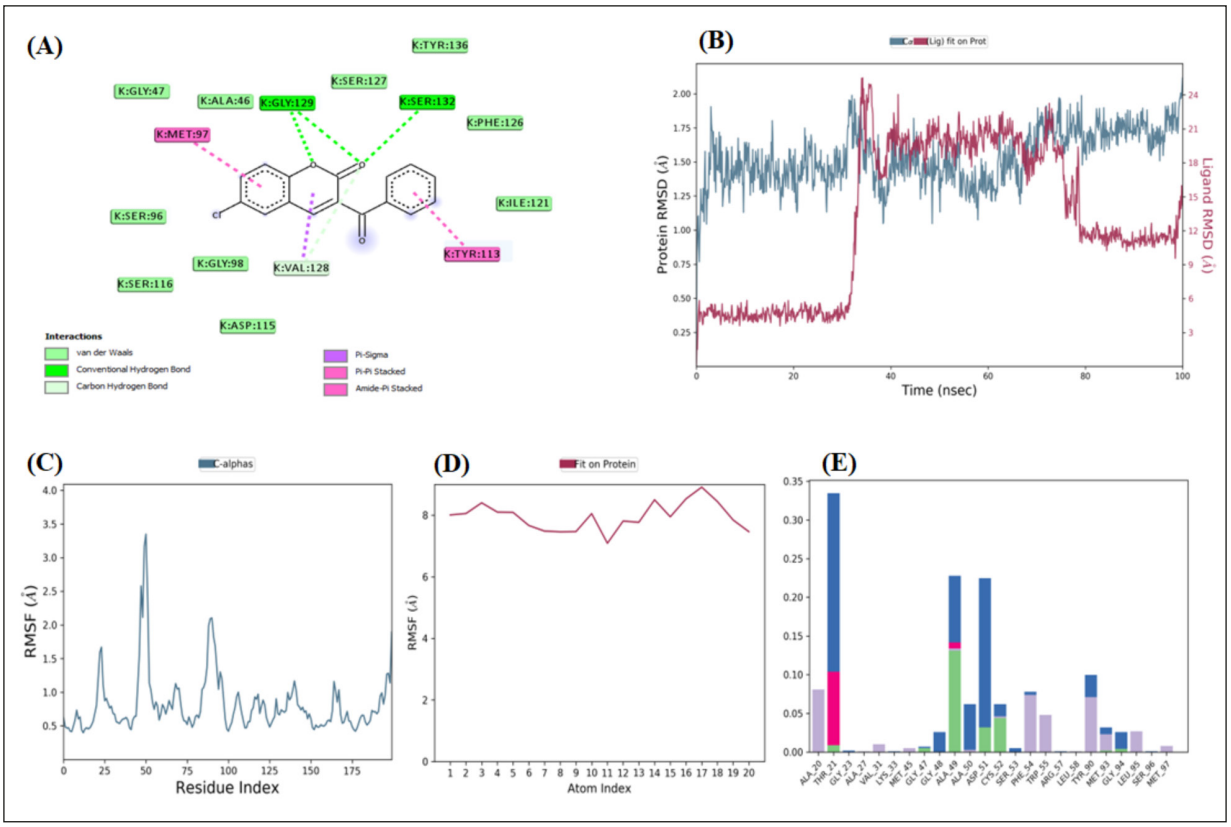

Figure 4. The results of molecular docking and MD simulation of the compound US-I-64 with the proteasome. (**A**), two-dimensional interaction obtained after docking into proteasome. (**B**), RMSD of the proteasome and US-I-64 complex. (**C**), RMSF of protein backbone. (**D**), RMSF of the ligand with respect to protein. (**E**), Protein ligand interactionsobserved after MD simulation.

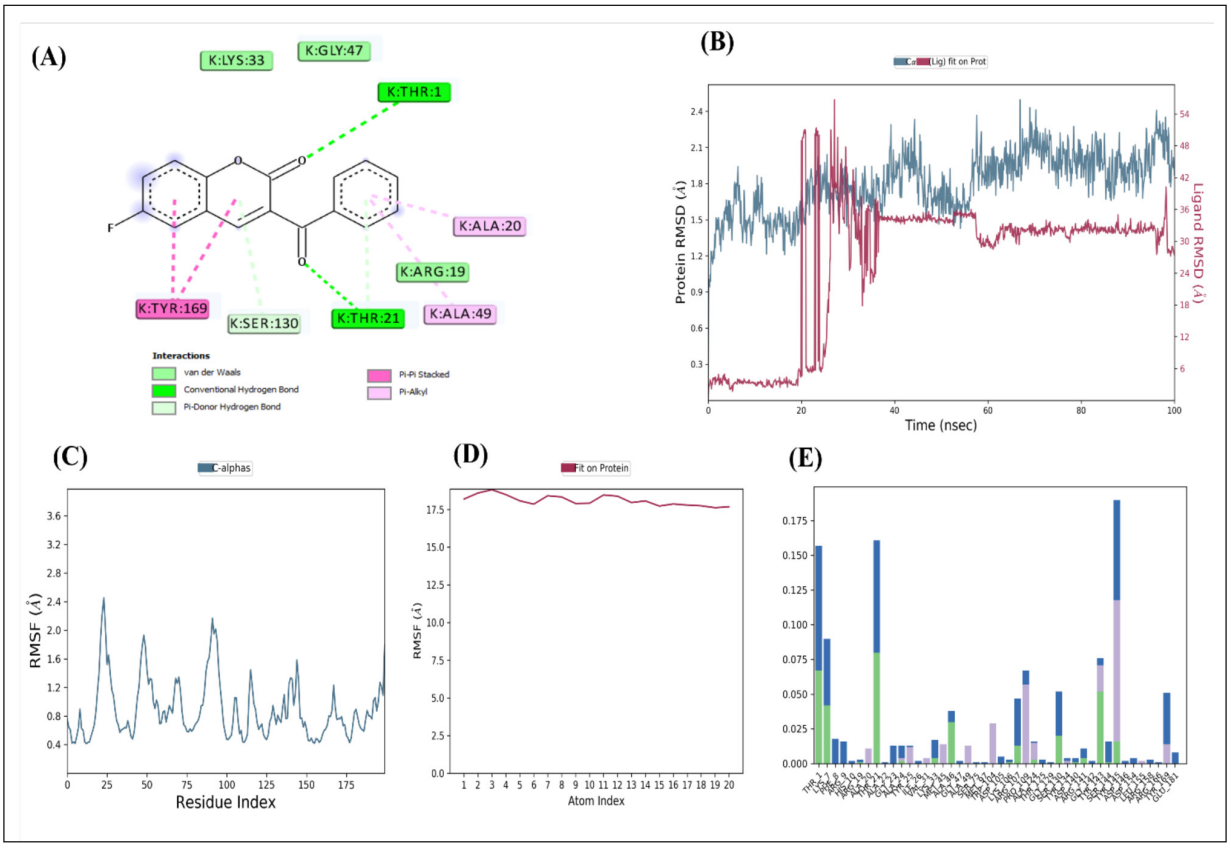

Figure 5. The results of molecular docking and MD simulation of the compound US-I-66 with the proteasome. (**A**), two-dimensional interaction obtained after docking. (**B**), RMSD of the protein-ligand complex. (**C**) RMSF of protein backbone. (**D**), RMSF of the ligand with respect to protein. (**E**), Protein ligand interactions observed after running MD simulation for 100 ns.

Molecular Dynamics Simulations

Due to the unavailability of proteasomal protease in purified form, the binding of these compounds was validated by performing MD simulations for 100 ns in triplicates using the Desmond Simulation Package. The results of MD simulation were reproducible for each of the four complexes. As suggested by the interaction pattern between the proteasome and US-I-64 compound (Figure 4A), the RMSD plot of the complex (Figure 4B) showed that the complex was shortly stable, i.e., after 33nsthe ligand RMSD increased to 24 Å and showed fluctuations till the end of the simulation. RMSF plot of protein residues also showed fluctuations (Figure 4C). Additionally, the ligand RMSF was also high, i.e., 8 Å (Figure 4D). The protein-ligand interactions showed the N-terminal threonine did not form any type of bond in the whole course of the simulation (Figure 4E).

Contrary to the observed interaction pattern between the proteasome and US-I-66 compound (Figure 5A), the RMSD of proteasome US-I-66 complex was only stable till 20 ns, and after that, extreme fluctuations were observed. Moreover, the ligand RMSD also went higher, i.e., up to 54 Å (Figure 5B). The RMSF of the protein backbone was found to be under 2.4 Å, but fluctuations were also visible (Figure 5C). The ligand RMSF was observed to be extremely high i.e., 17.5 Å (Figure 5D). The main catalytic residue Thr1 was involved in the formation of hydrogen bonds and hydrophobic bonds, and the amino acid residue Lys33 also formed interactions with the protein (Figure 5E), but these interactions were for a very short period i.e., only 15% and 2.2% respectively.

As expected from the interaction analysis between the proteasome and the US-I-67 compound (Figure 6A), the proteasome US-I-67 complex was very unstable, and extreme fluctuations in ligand RMSD were observed (Figure 6B). The RMSD of the ligand was observed to be very high at 80 Å at the beginning of the simulation, and after attaining stability, the RMSD was observed to be 40 Å, which shows the instability of the complex. When the RMSF of the protein was assessed, it was observed that some of the residues

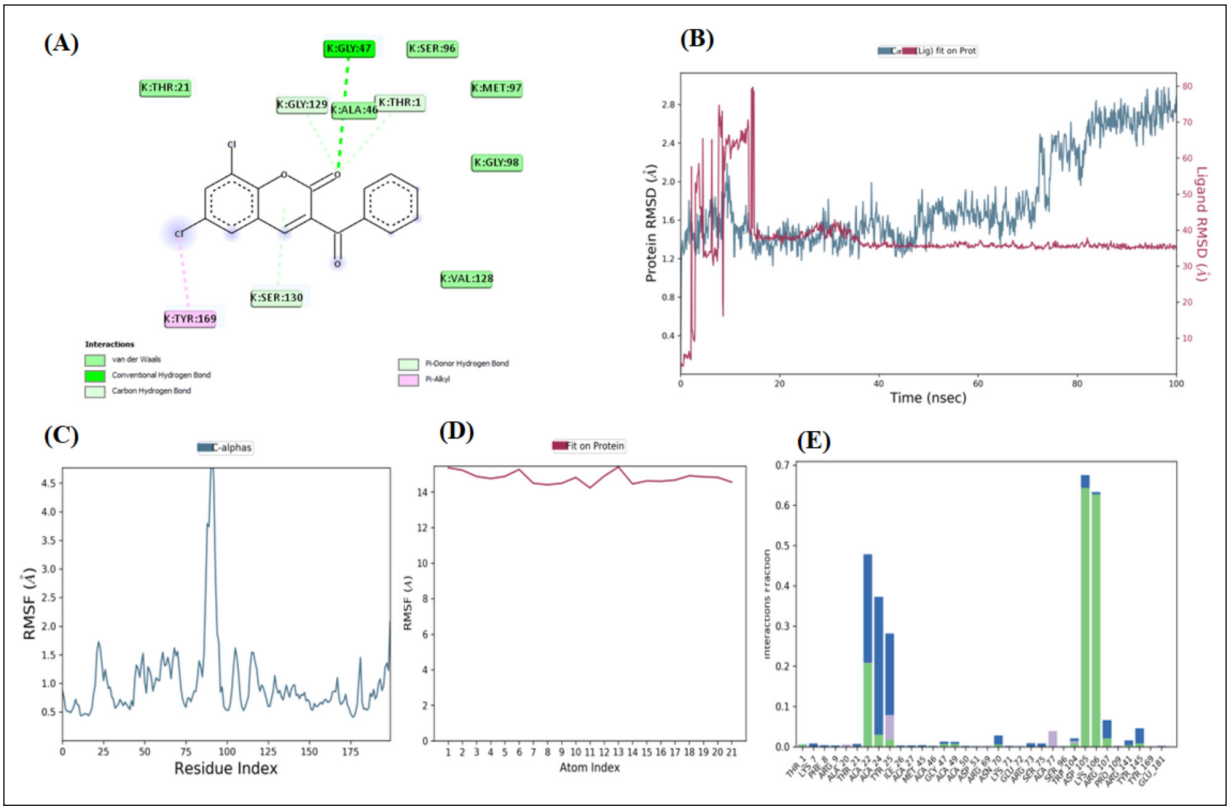

Figure 6. The results of molecular docking and MD simulation of the compound US-I-67 with the proteasome. (**A**), Two-dimensional interaction of the docked US-I-66 into the active site of the proteasome. (**B**), RMSD of the proteasome and US-I-67 complex. (**C**), RMSF of protein backbone. (**D**), RMSF of the ligand with respect to protein. (**E**), Protein-ligand interactions observed after MD simulation.

showed a higher value, and most of the residues showed an RMSF of less than 2 Å (Figure 6C). However, the ligand RMSF was found to be comparatively higher than the protein i.e., 15 Å (Figure 6D). The overall protein-ligand interactions were minimal, and only a few interactions were observed. The main catalytic residue Thr1 was observed to be involved in the formation of Hydrogen bonds with the ligand but only for an extremely small period i.e., only 1% of the total simulation (Figure 6E).

As depicted from the proteasome and US-I-68 interaction analysis (Figure 7A), the RMSD plot of the proteasome-US-I-68 complex showed a great number of fluctuations throughout the simulation, and RMSD was observed to increase up to 56Å (Figure 7B). The RMSF of most of the amino acid residues was below 2Å (Figure 7C), but the ligand RMSF was quite higher i.e., 17 Å (Figure 7D). The amino acid residue Thr1 formed water bridges with the protein only for 0.3% simulation time. Moreover, significant hydrogen bonds were not observed with any of the residues (Figure 7E). The results obtained from MD simulations of all four complexes revealed that none of the compounds formed a stable complex with the proteasome, and thus, these results indicate that these compounds are selective inhibitors of the HslV protease.

The HslV Protease Inhibition Assay

Among all docked compounds, those showing the highest docking scores were further tested for their inhibitory potential against the HslV protease using an *in-vitro* assay. The outcomes of enzyme inhibition assays were in accordance with the molecular docking results. The dose-response is represented in the form of percent inhibition exhibited by each compound in a concentration-dependent manner (Figure 8). The results of the enzyme inhibition assay are in accordance with the molecular docking results. The IC_{50} of all the compounds has been presented in Table I.

During the enzyme inhibition assay, the compound US-I-67 exhibited the highest inhibitory potential against the HslV protease with an IC_{50} value of 0.27 ± 0.01 µM. The compounds US-I-66

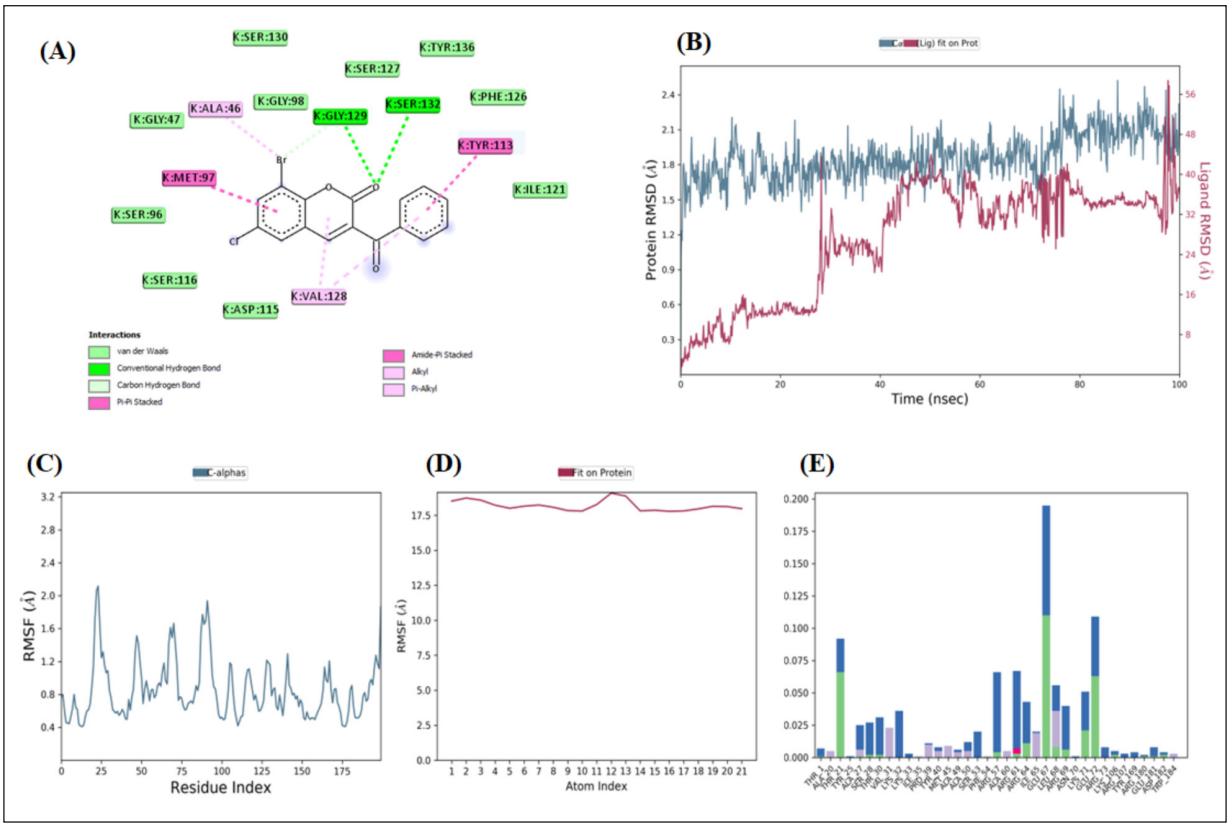

Figure 7. The results of molecular docking and MD simulation of the compound US-I-68 with proteasome. (**A**), Two-dimensional interaction obtained after docking into the active site of the proteasome. (**B**), RMSD of the proteasome and US-I-68 complex. (**C**), RMSF of protein backbone. (**D**), RMSF of the ligand with respect to protein. (**E**), Protein-ligand interactions observed after MD simulation.

and US-I-68 also showed good inhibition with IC_{50} = 0.62 ± 0.011 and 0.44 ± 0.026 µM, respectively. The compound US-I-64 exhibited the lowest inhibitory potential with an IC_{50} = 0.73 ± 0.029 µM among all the tested compounds.

Discussion

The HslVU complex, along with other proteases, is involved in the regulation of proteostasis by degradation of misfolded and incomplete polypeptides[3]. Due to the global increase in antibiotic resistance, the development of new antibacterial drugs having novel modes of action is a need of the hour. Since the ATP-dependent proteases play a critical role in the degradation of misfolded proteins and the maintenance of overall cell homeostasis, they are considered attractive drug targets[54,55]. Many studies[15-17] regarding the inhibition of bacterial proteases have been reported. Despite its significant role, the HslVU complex has been neglected in the past. In the last decade, HslV protease was targeted using a different strategy, i.e., by identifying small chemical molecules that could over-activate this protease[46]. Herein, we have manipulated the HslVU inhibition to identify effective antimicrobial drug candidates.

To identify potential inhibitors of the HslV protease, the active three-dimensional structure was a prerequisite. As the active *E. coli* HslVU structure having HslU C-tail intercalated into the pocket between two adjacent HslV subunits along with the correct active site architecture was missing in PDB, a homology model of *E. coli* HslVU complex was built using the crystal structure of the *H. influenzae* HslVU complex as a template. The homology model was built in a complex with the inhibitor NLVS. The built model was observed to be of good quality both in terms of internal energy and overall stereochemistry. The superposition of both structures revealed that the generated model of the *E. coli* HslV protease resembled the three-dimensional structure of *H. influenzae* HslV protease, including the active site architecture.

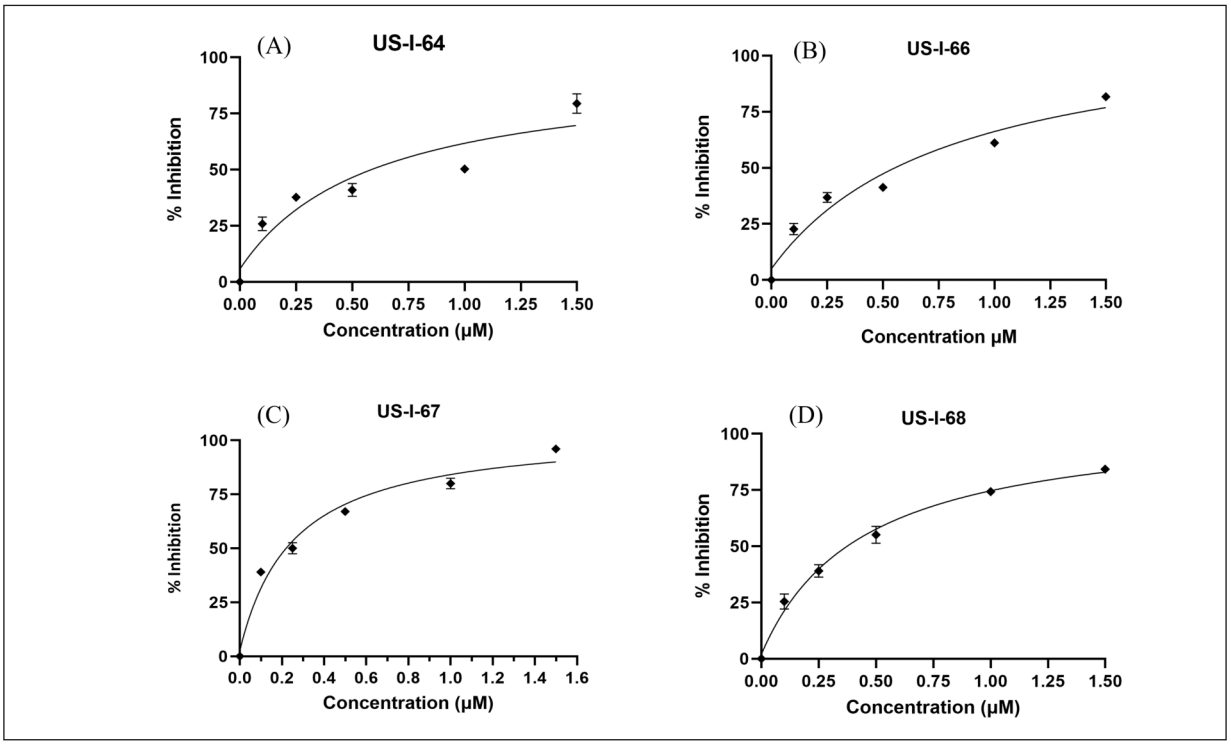

Figure 8. The results of the *E. coli* HslV protease inhibition assay. Percent inhibition of each compound (**A**), US-I-64, (**B**), US-I-66, (**C**), US-I-67, (**D**), US-I-68 is plotted as a function of inhibitor concentration. The data points represent the mean values and standard deviations.

Coumarins are considered a medicinally important class of compounds and have shown anti-bacterial, anti-leishmanial, anti-cancer, and anti-viral effects in several studies[19-21,56]. In the present study, twenty-three derivatives of the 3-substituted coumarin class were selected for evaluating their inhibitory effects on the HslVU protease/chaperone complex by both *in-silico* and *in-vitro* studies. The selected derivatives include 3-benzoyl, 3-acetyl, and 3-ethyl ester derivatives of coumarin. Among all the compounds, the 3-benzoyl derivatives showed the highest docking scores compared to the acetyl and ethyl ester derivatives. Out of the eleven 3-benzoyl derivatives, those having chloro, fluoro, and bromo substitutions showed more potential to inhibit the HslVU complex. A detailed analysis of the results of molecular docking studies showed that among all the docked compounds, the compound US-I-67 formed the highest number of interactions with the HslV binding site. This large number of interactions is likely a contributing factor to its highest docking score. The role of Tyr161 remained unclear, but the present study pointed toward its important role in the binding mechanism of the HslV protease, as it was also found to interact with the known inhibitor NLVS. A comprehensive analysis of the two-dimensional and three-dimensional docked poses of these four ligands revealed that all the 3-substituted coumarin derivatives were bound to the active site of *E. coli* HslV protease with their benzoyl group inserted inward while the other part of the ligand remained outside. Notably, the presence of additional halogen substitutions in these compounds showed enhanced docking scores and better IC_{50} values.

Since the HslV protease is considered a homolog of the eukaryotic 20S proteasome, the inhibitory potential of these compounds against 20S proteasome was also evaluated by a detailed *in-silico* approach. The chymotrypsin-like activity of proteasome was targeted for inhibition since it is important, and most of the reported proteasome inhibitors act by mainly inhibiting this activity[53]. These four identified inhibitors of the HslVU protease/chaperone complex did not show meaningful interactions with the proteasomal active site. Due to the unavailability of proteasomal protease in purified form, the binding of these compounds was validated by performing MD simulations for 100 ns in triplicates. The results of MD simulations demonstrated that all the docked

complexes between these lead compounds and the proteasome were quite unstable. The trajectories of MD simulations and protein-ligand contacts also validated that these compounds do not have the potential to inhibit proteasome.

A direct correlation was observed between docking scores and IC_{50} values of the tested compounds. The inhibitory potential of the tested compounds against the HslV protease was found to be in this order US-I-67>US-I-68>US-I-66>US-I-64. The highest docking score and the most stable interaction observed between the compound US-I-67 and HslV corroborate its highest inhibitory potential.

Based on the obtained results, we believe that these small chemical molecules can inhibit bacterial growth by blocking one of the important members of their proteolytic machinery without affecting the host 20S proteasome.

Conclusions

The HslVU complex, along with other proteases, is involved in the regulation of proteostasis by degradation of misfolded and incomplete polypeptides. Considering the worldwide surge in antibiotic resistance, the ad-vent of new antibacterial with unique mechanisms of action is a need of the hour. The crucial role of the HslV protease in the regulation of proteostasis and modulation of heat-shock response makes it a promising drug target, but it has not been explored till now. Here, we report the inhibitory potential of four 3-substituted coumarin derivatives, including 6-chloro-3-(phenylcarbonyl)-2H-chromen-2-one (US-I-64), 6-fluoro-3-(phenylcarbonyl) -2H-chromen-2-one (US-I-66), 6,8-dichloro-3-(phenylcarbonyl)-2H-chromen-2-one (US-I-67) and 8-bromo-6-chloro-3-(phenylcarbonyl)-2H-chromen-2-one (US-I-68) against the HslV protease. Here, we believe these lead molecules will have a lethal effect on bacteria as they tend to disturb their overall proteostasis at very low doses without affecting the host proteostasis.

Authors' Contributions

Conceptualization, M.H, Y.R, M.K.A, K.M.K, T.A; methodology, M.H, U.S, Y.R, M.K.A, K.M.K, T.A; software, M.A; validation, A.A.S; formal analysis, A.F.A.; investigation, M.H, U.S, Y.R, M.K.A, K.M.K, T.A; resources, M.A and A.A.S.; data curation, A.F.A, M.H; writing—original draft preparation, M.H. T.A and Y.R.; writing—review and editing, M.H, M.K.A, K.M.K, S.N and U.S; visualization, A.F.A, A.S; supervision, Y.R and T.A; project administration, A.A.S and M.A; funding acquisition, T.A.

Conflicts of Interest

The authors declare no conflict of interest.

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Ethics Approval

Not applicable as no human or animal samples were included in this study.

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