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Inhibitory potential of triazines and hydrazinyl thiazole substituted chromones against the HslVU protease/chaperone complex, a novel drug target

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Abstract. – OBJECTIVE: Proteostasis is an important process occurring in all living cells and is highly indispensable for cell survival. The HslVU protease/chaperone complex's critical role in regulating proteostasis to maintain a healthy cellular proteome and its presence in pathogenic microbes made it an important drug target. This study aimed to identify small molecular inhibitors of the HslV protease.

MATERIALS AND METHODS: Herein, a library of small molecules belonging to the triazine and chromone families has been evaluated for their inhibitory potential against the *E. coli* HslV protease using both *in silico* and *in vitro* techniques.

RESULTS: Four compounds, i.e., SHS-II-123a, SHS-II-147a, US-IV-89, and US-IV-92, were identified as potential inhibitors of the HslV protease having IC_{50} values in the range of 0.1 to 0.32 µM. Additionally, these compounds' drug-likeness and ADMET profiles indicated their compatibility to be considered safer drug candidates.

CONCLUSIONS: To the best of our knowledge, this is the first report on small molecules having inhibitory effects on the HslVU complex. These identified compounds can be efficiently subjected to further investigations to develop novel and safer antimicrobial agents.

Key Words:
The HslVU complex, Proteostasis, Triazine, Chromone, IC_{50}, HslV inhibitors.

Introduction

Regulated proteostasis is an essential process for the viability of living cells. It involves the degradation of non-native or misfolded proteins inside the cell, preventing their accumulation and protecting the cell from possible damage. Many ATP-dependent proteases, including the HslVU protease/chaperone complex, have been identified in bacteria, which regulate proteostasis. These proteases regulate various stress responses, including heat shock, DNA damage, and oxidative stress[1]. The HslVU complex is encoded by the *hslVU* operon and consists of two subunits, i.e., the HslV protease and the HslU ATPase[2,3]. The central core of the HslVU complex is made up of proteolytic HslV dodecamer flanked by two HslU hexamers. The HslV protease has weak peptidase activity and only degrades certain unfolded proteins such as casein. Its proteolytic activity is strikingly enhanced by its counterpart, *i.e.,* HslU, in the presence of ATP[4]. Additionally, the degradation of proteins by the HslV protease requires unfolding and translocation to its proteolytic chamber, which is achieved by HslU[5,6].

The HslVU complex is involved in the degradation of important regulatory proteins, including σ^{32}, the heat shock factor[7]; SulA, a cell-division inhibitor[8], RcsA, the transcriptional regulatory protein[9], TraJ the transcriptional activator of F transfer operon[10], and Arc repressor[11]. This HslVU complex is a very important entity of the cell as it equally contributes to the regulation of protein homeostasis along with other cellular ATP-dependent proteases and promotes the resistance to cellular stress and thus promotes survival[12].

Due to the rapidly growing bacterial resistance towards classical antibiotics, there is an urgent

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need to develop new therapeutic strategies to cope with several life-threatening bacterial infections. The significant role of the HslVU complex in many pathogens is being acknowledged day by day. It is essential for the survival of *Salmonella Enterica* inside the host[13] as well as involved in its attachment to the host's surface[14]. In *Shewanella oneidensis*, an aquatic model bacterium, the HslVU protease has been identified as a potential member of the proteostasis network, where it tends to regulate the level of TilS, involved in tRNA modification[15]. Recently, the HslVU has been reported to promote bacterial survival during the phase of growth arrest in another pathogenic bacteria, *Pseudomonas aeruginosa*[16]. Besides bacteria, the HslVU complex is present in several pathogenic microorganisms, including *Trypanosoma cruzi*, *Leishmania,* and *Plasmodium falciparum*[17-20]. Due to the critical role of HslV in regulating proteostasis in the living cell to maintain a healthy cellular proteome, and its presence in pathogenic microbes the HslV protease is considered an important drug target for the development of new therapeutic agents against these pathogenic microbes. This study evaluated a library of small molecules belonging to the triazine and chromone family for their inhibitory potential against the HslV protease.

Materials and Methods

Molecular Docking Studies

Homology modeling of *E. coli* HslV (EcV) was carried out using MODELLER V9.15[21] to obtain the optimum conformation of receptor protein for ligand docking studies. For molecular docking studies, twenty-six derivatives of hydrazinyl thiazole substituted chromones and twenty-four derivatives of triazines were selected and their three-dimensional structures were sketched and optimized using Marvin Sketch[22]. AutoDock tools[23] prepared the PDBQT files for the protein and ligands. This preparation includes the addition of polar hydrogens, removal of heteroatoms, and addition of charges. All the selected compounds were docked in the active site of the EcV using AutoDock Vina (1.1.2) software[24]. The docking results were expressed in terms of docking scores in kcal/mol. Analysis of docking results was carried out using LigPlot (version 2.2.4)[25] and DS-Visualizer software (version 16.1.0.15350)[26]. Compounds showing the highest docking scores were selected for further *in vitro* analysis.

ADMET Profiling

The ADMET properties and drug-likeness of the compounds were calculated by SwissADME (http://www.swissadme.ch/index.php)[27] and pkCSM (http://biosig.unimelb.edu.au/pkcsm/ Link)[28] online servers.

HslV Protease Inhibition Assay

The pET12b+ vector containing *E. coli* HslV with a C-terminal 6xHis-tag and pET22b vector containing *E. coli* HslU with N-terminal 6xHis-tag were transformed into *E. coli* BL21-Codon Plus (DE3)-RIL cells (Agilent, Santa Clara, CA, USA) for protein expression. HslV and HslU proteins were expressed and purified as previously described[29]. The expression and purification of both the proteins were confirmed by running SDS gels. The purified proteins were quantified by measuring the absorbance at 280 nm. Four compounds *i.e.,* SHS-II-123a, SHS-II-147a, US-IV-89 and US-IV-92, showing the best docking scores and interactions with the HslV protease were assessed for their *in vitro* inhibition potential at 0.1, 0.25, 0.5, 1.0 and 1.5 µM concentrations. These compounds were obtained from the Compound Bank, ICCBS, University of Karachi, which contains a variety of isolated/synthetic chemical compounds in purified forms. The procedure of compound synthesis and associated data has already been published[30,31].

Peptide hydrolysis was assayed by incubating HslU (10 nM), HslV (5 nM), Tris-HCl buffer (pH 8) containing 5 mM $MgCl_2$, 2 mM ATP, 0.5 mM EDTA and 0.1 mM Z-Gly-Gly-Leu-AMC (Sigma Aldrich, Saint Louis, MA, USA) and 5% DMSO and the respective compound as described previously[32]. The assay was performed in a 96-well plate with a 0.1 ml reaction mixture. The standard inhibitor NLVS (20 µM) was used as the reference inhibitor. Inhibition of HslV was evaluated by continuously measuring the fluorescence (λ_{ex} = 355 nm, λ_{em} = 460 nm) of released AMC from fluorogenic peptide substrate Z-GGL-AMC at 37°C using Varioskan LUX microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). Each experiment was conducted in independent triplicates. The percent inhibition of HslV protease was calculated according to the given equation: % Inhibition = 100 x [1-(X-min)/(max-min)]. IC_{50} was calculated from the graph plotted *vs.* compound concentration and percent inhibition. Standard statistical protocols calculated standard deviation (SD) and Standard error of the mean (SEM).

Results

Molecular Docking Studies

Molecular docking is one of the widely used methods in structure-based drug design, and several studies have been carried out to identify drug candidates against different pathogens[33-35]. The three-dimensional model structure of *E. coli* HslV protease complexed with NLVS is shown in Figure 1a. Virtual screening of fifty derivatives was performed to identify potential lead molecules that can effectively inhibit *E. coli* HslV protease. NLVS, the standard inhibitor, was used as a reference molecule[36]. The structure and docking

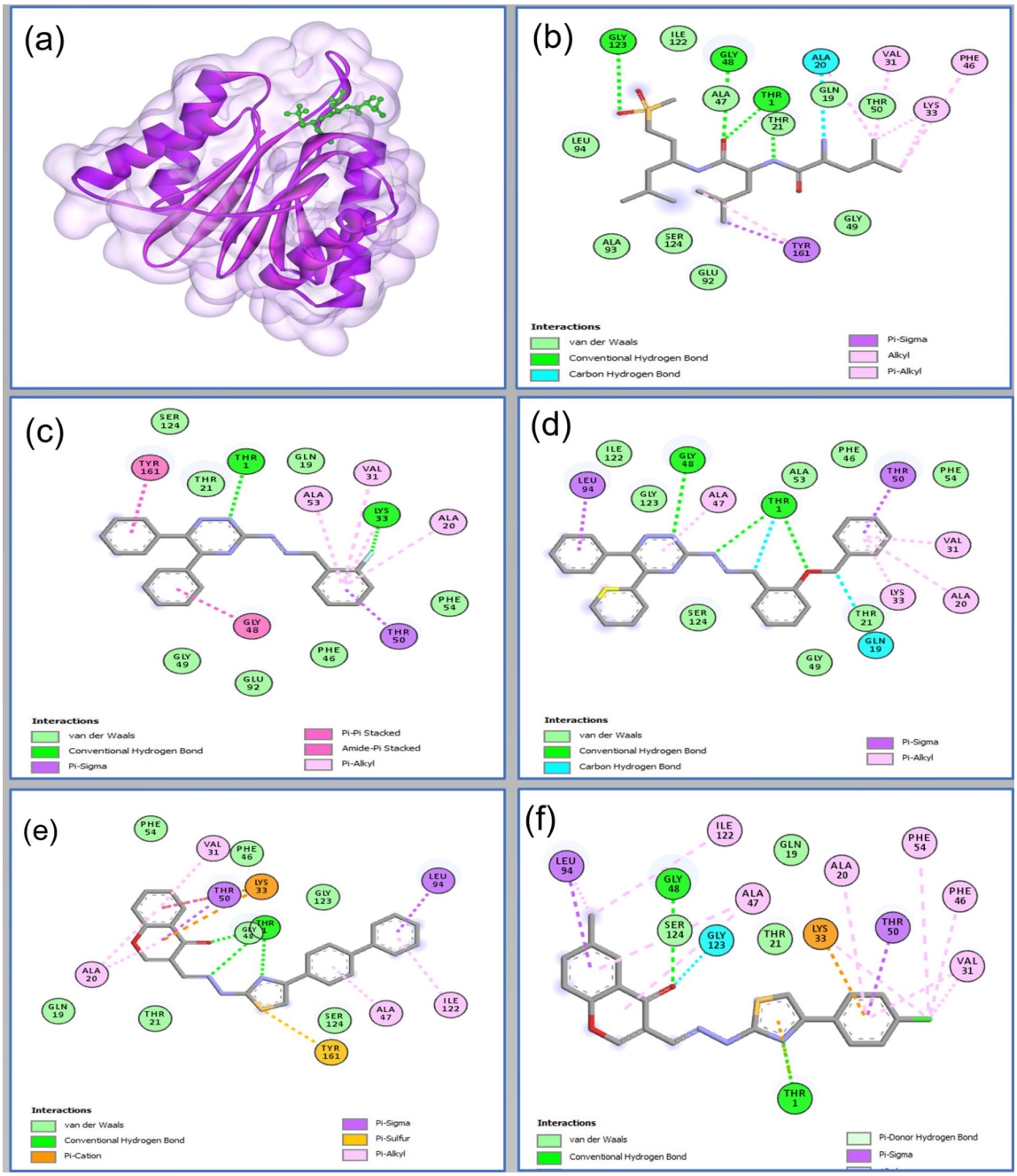

Figure 1. Molecular docking studies against *E. coli* HslV protease. Three-dimensional modelled structure of *E. coli* HslV protease with bound NLVS (*green*) is represented (**a**); Two-dimensional representation of EcV interactions with NLVS after docking is also shown (**b**). Two-dimensional representations of protein-ligand interactions between EcV and the top scoring ligands including SHS-II-123a (**c**), SHS-II-147a (**d**), US-IV-89 (**e**) and US-IV-92 (**f**) are also depicted.

scores of all the docked compounds are listed in Table I. High docking scores (more negative values) indicate a stronger enzyme-ligand interaction while the lower docking scores (lesser negative values) express a weaker interaction between the ligand and the enzyme. Some of the compounds showed a lesser docking score than NLVS but most of the docked compounds showed higher docking scores. The docking score for NLVS was found to be -6.3 kcal/mol. Four compounds including SHS-II-123a, SHS-II-147a, US-IV-89, and US-IV-92 showed the highest docking scores of -9.0 kcal/mol, -9.2 kcal/mol, -8.5 kcal/mol, and -8.7 kcal/mol, respectively (Table I). The docking scores and the structure of all the docked compounds are provided in **Supplementary Table I**.

The two-dimensional interactions of the best-docked compounds have also been displayed in Figure 1. The receptor-ligand interactions were primarily built up by forming hydrogen bonds with the main catalytic residue Thr1 (shown by green dashed lines). The Oγ atom of catalytic N-terminal threonine of EcV, which serves as the nucleophile and forms a covalent bond with NLVS in the crystal structure, was found to form conventional hydrogen bonds with NLVS (Figure 1b). The amino acid residue Lys33, which also plays a key role in the catalysis, was involved in the formation of a hydrogen bond with the triazine derivative SHS-II-123a (Figure 1c), Pi-alkyl (pink color) bond with SHS-II-147a (Figure 1d) and Pi-cation (brown color) with the chromone derivatives US-IV-89 (Figure 1e) and US-IV-92 (Figure 1f). Pi-sigma interactions (purple dashed lines) were also formed with Thr50. The residue Val31 formed Pi-alkyl bonds (pink color) with all the compounds. Since all these Pi interactions participate in charge transfer, therefore, play key role in aligning the protein and the ligands. The other important residues, Thr21, Gly48, Thr50, and Ser124 were also involved in multiple interactions with the enzyme in all four compounds. The protein-ligand complex was further stabilized by van der Waals interactions with residues i.e., Gln19, Gly23, Phe46, Phe54. The receptor-ligand interaction of these compounds with the active site of the HslV protease was quite similar to that observed in the case of NLVS.

ADMET Profile and Drug-Likeness

The drug-likeness, pharmacokinetics, and ADMET properties of the compounds were also evaluated. The predicted drug-likeness properties of the identified lead molecules are displayed in Table II. Lipinski's rule of five was followed by all the compounds. Furthermore, other physiochemical properties of the compounds were also found to be in the appropriate range. The topological polar surface area (TPSA) of a molecule linked with a drug's bioavailability is a particularly important property in the drug discovery process. All these compounds showed TPSA values lower than 140 Å² which is needed for the good bioavailability of a drug. Moreover, the measure of lipophilicity consensus the Log $P_{o/w}$ value which should not exceed five has been observed in the desired range for all these lead molecules.

Table I. Molecular docking scores and IC 50 values of the compounds with topmost docking scores.

Compound Codes	IUPAC names	Binding energy with HslV (Kcal/mol)	IC_{50} ± SEM (μM)
NLVS	(2S)-2-[(2S)-2-[2-(4-hydroxy-3-iodo-5-nitrophenyl)acetamido]-4-methylpentanamido]-N-[(1E,3S)-1-methanesulfonyl-5-methylhex-1-en-3-yl]-4-methylpentanamide	-6.3	12 ± 0.22
SHS-II-123a	3-[(E)-2-[(2-fluorophenyl)methylidene]hydrazin-1-yl]-5,6-diphenyl-1,2,4-triazine	-8.4	0.13 ± 0.005
SHS-II-147a	3-[(E)-2-{[2-(benzyloxy)phenyl]methylidene} hydrazin-1-yl]-5,6-diphenyl-1,2,4-triazine	-8.6	0.1 ± 0.005
US-IV-89	(E)-3-((2-(4-(biphenyl-4-yl)thiazol-2-yl) hydrazono)methyl)-4H-chromen-4-one	-8.0	0.29 ± 0.01
US-IV-92	(E)-3-((2-(4-(4-chlorophenyl)thiazol-2-yl) hydrazono)methyl)-6-methyl-4H-chromen-4-one	-8.3	0.32 ±0.015

Table II. The compounds also exhibited overall good ADMET properties.

Property	SHS-II-123a	SHS-II-147a	US-IV-89	US-IV-92
Drug likeness				
Lipinski's rule of five	Yes	Yes	Yes	Yes
Bioavailability	0.55	0.55	0.55	0.55
Consensus Log $P_{o/w}$	4.36	5.26	5.03	4.58
No. of H-bond acceptors	5	5	4	4
No. of H-bond donors	1	1	1	1
No. of rotatable bonds	5	8	5	4
TPSA	63.06 Å	72.29 Å	95.73 Å	95.73 Å
Absorption				
Caco2 permeability (log Papp in 10-6 cm/s)	1.098	1.105	0.31	0.577
Intestinal absorption	93.98%	94.2%	91.409%	90.165%
Water solubility (log mol/L)	-3.923	-3.162	-6.341	-5.003
Skin permeability (log Kp)	-2.735	-2.735	-2.734	-2.67
P-glycoprotein substrate	Yes	No	Yes	Yes
Distribution				
VDss (log L/Kg)	-0.309	-0.628	0.048	0.181
BBB permeant	Yes	No	No	No
CNS permeability	-1.897	-1.788	-1.468	-1.607
Metabolism				
CYP2D6 substrate	No	No	No	No
CYP3A4 substrate	Yes	Yes	Yes	Yes
CYP1A2 inhibitor	Yes	No	Yes	Yes
CYP2C19 inhibitor	Yes	Yes	Yes	Yes
CYP2C9 inhibitor	Yes	Yes	Yes	Yes
CYP2D6 inhibitor	No	No	No	No
CYP3A4 inhibitor	Yes	Yes	Yes	Yes
Excretion				
Renal OCT2 substrate	No	No	No	No
Toxicity				
AMES toxicity	No	No	No	Yes
Max. tolerated dose (human)	0.814	0.543	0.73	0.394
Skin sensitization	No	No	No	No
Hepatotoxicity	Yes	Yes	Yes	Yes

The compounds also exhibited overall good ADMET properties (Table II). The important evaluated properties are Caco2 permeability (recommended range log Papp> 8 x 10^{-6} cm/s), intestinal absorption, water solubility, cytochrome P450 interaction, skin permeability, and Volume of distribution at steady states (VDss) were found to be within recommended ranges. All these compounds were found to be safe for the central nervous system (CNS) and do not have the potential to cross blood brain barrier (BBB). These compounds when checked for toxicity evaluation they did not show toxicity. AMES toxicity is widely evaluated to check the mutagenicity of any compound to analyze whether it can be carcinogenic or not. Among these four compounds, only one compound US-IV-92 showed AMES toxicity potential. The maximum tolerated dose of these compounds was also estimated and was found to be admissible.

The HslV Protease Inhibition Assay

The HslV protease and the HslU ATPase gave high expression and were purified successfully. The HslV protease inhibition assay was carried out using the best-docked compounds. The percent inhibition represents the inhibition in a concentration-dependent manner (Figure 2). The results of the enzyme inhibition assay were found to be consistent with the molecular docking results. All four tested compounds showed significant inhibitory potential in the fluorescence-based inhibition assay. The triazine derivative SHS-II-147 has shown the highest inhibition against the HslV protease with an IC_{50} value of only 0.1 ± 0.005 µM (Table I). The other compounds also showed significant inhibitory activity; SHS-II-123a (IC_{50} = 0.13 ± 0.005), US-IV-89 (IC_{50} = 0.29 ± 0.01 µM) US-IV-92 (IC_{50} = 0.325 ± 0.015 µM).

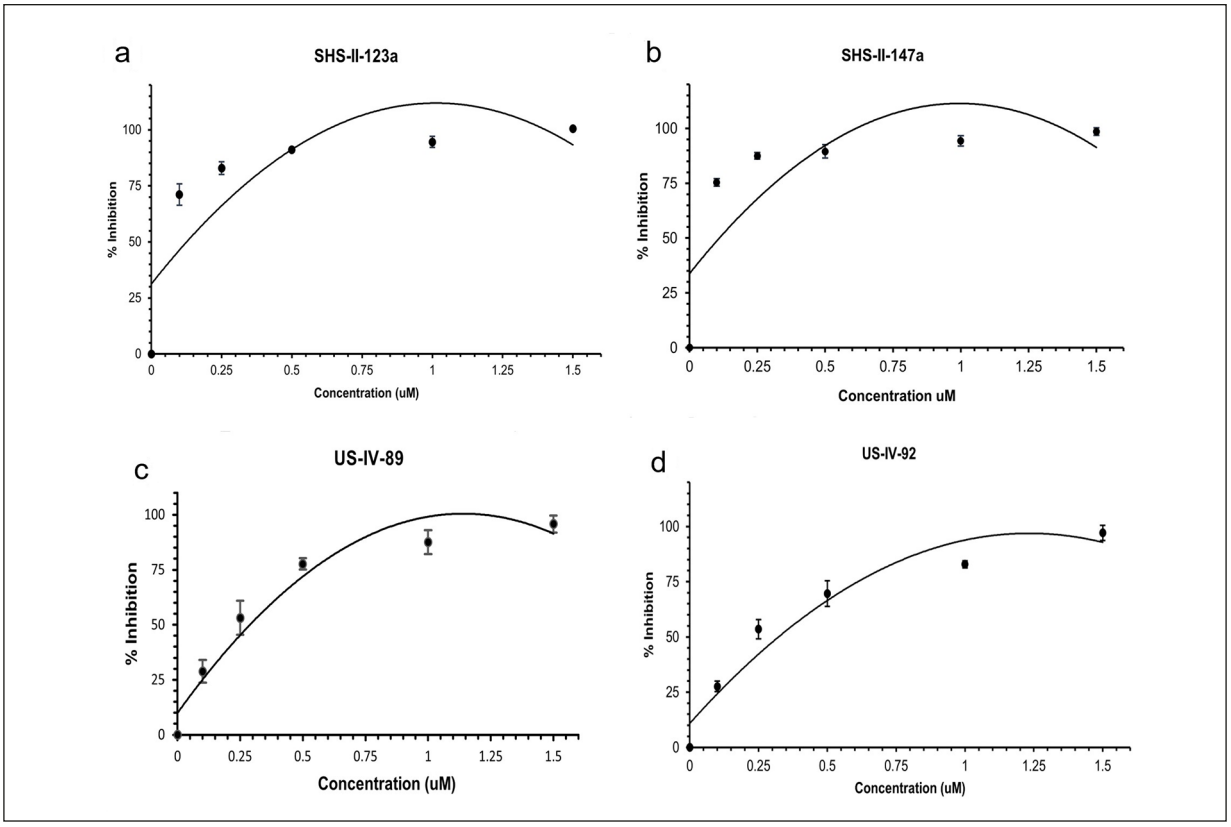

Figure 2. The HslVU protease/chaperon complex inhibition assay. The percent inhibition of each compound, i.e., (**a**) SHS-II-123a (**b**) SHS-II-147a (**c**) US-IV-89 (**d**) US-IV-92, was plotted as a function of inhibitor concentration. The data points represent the averages and standard deviations of measurements from independent triplicate wells.

Discussion

Proteostasis is one of the important processes and it is highly indispensable for the survival of the cell. It involves the energy-dependent degradation of misfolded and unwanted proteins to maintain the healthy proteome of the cell. In bacteria, this process is primarily carried out by AAA+ proteases[37]. The HslVU complex is one of such proteases present in bacteria and found to be involved in the degradation of misfolded and abnormal polypeptides[2]. Due to the growing antibiotic resistance around the globe, developing new antibacterial drugs with novel modes of action has become necessary. The bacterial infection is often characterized by increased body temperature and is considered a defensive measure, which in turn triggers the heat shock of the invading pathogen[38]. This heat shock induces the expression of heat shock proteins to cope with this adverse condition. The AAA+ proteases regulate the protein quality control by degradation of proteins damaged by the heat and other stresses and maintain the overall cell homeostasis. Thus, they are considered attractive drug targets for developing antibiotic drugs[39,40].

Small molecules are particularly important in drug discovery because of their lower molecular weights and higher cellular permeability[41]. Here we selected triazines and chromones derivatives to evaluate the HslV protease inhibition potential. Triazine is an important class of heterocyclic compounds, and its derivatives have antifungal, antimicrobial, anticancer, and anti-inflammatory activities[30]. Similarly, chromones derivatives have significant importance in drug discovery. Their antioxidant, antibacterial, anti-inflammatory, anticancer, and antiviral activities have already been reported[31]. In this study, twenty-four derivatives of triazines and twenty-six derivatives of chromones were screened for the HslV protease inhibition using *in-silico* and *in-vitro* methods. The results of molecular docking studies were thoroughly analyzed to find out the best hits and the compounds showing the highest score and the most optimum interactions were selected for

in-vitro enzyme inhibition assay. In molecular docking studies, all these compounds formed hydrogen bonds with the first threonine, the main catalytic residue of the HslV protease. Significant interactions were also observed with the amino acid residue lysine[33]. These interactions and high docking scores account for these compounds' remarkable HslV inhibitory activity.

All the four tested compounds showed great inhibitory potential towards the HslV protease comparatively, the triazine derivatives showed higher inhibitory potential than the chromone derivatives. The results of the HslV protease inhibition assay were found to be in accordance with the results of molecular docking studies. The compounds showing the high docking scores also exhibited marked inhibition in the *in-vitro* assay.

Additionally, ADMET profiling of these compounds revealed good drug-likeness and pharmacokinetics properties. Since most of the absorption takes place in the small intestine, higher intestinal absorption is a legit descriptor of drug-likeness. All these compounds showed more than 90% intestinal absorption and lower skin permeability as well as good Caco2 permeability. These results revealed that these compounds would have great absorption properties. The distribution profile of these compounds was evaluated in terms of VDss, BBB, and CNS permeability. Except for the compound SHS-II-123a, all the compounds were not permeable to the brain. Blood Brain Barrier (BBB) prevents the entry of several molecules into the brain and protects the brain from possible harmful effects. The molecules having the potential to cross BBB may have adverse effects on the central nervous system. The compounds exhibited a suitable metabolic profile. Cytochrome P enzymes are the major drug metabolizing enzymes. Interestingly, all these compounds were neither observed as the substrates nor inhibitors of CYP2D6, a requirement for anti-depressants, antipsychotics, and other CNS drugs. CYP3A4 is an important enzyme of Phase I metabolism and is involved in the metabolism of approximately 50% of the drugs[42].

All the four tested compounds were found to be CYP3A4 substrates. In toxicity estimation, these compounds were observed to be safer drug candidates. Many drug candidates fail at the initial stages due to their toxicity and inappropriate ADMET profiles and cannot reach clinical trials. Therefore, the assessment of ADMET properties of lead molecules has become essential. In this study, the significant pharmacokinetic properties of these compounds suggest that they are excellent drug candidates and can be used in clinical trials in further studies.

The results of molecular docking studies, ADMET prediction, and the HslV inhibition assay indicate that these compounds are promising anti-bacterial and antimicrobial drug candidates and further *in vivo* activities should be conducted on these compounds for drug development.

Conclusions

Using HslV inhibitors as antibacterial drug candidates is a novel and efficient approach in treating several bacterial infections. The significant role of HslV protease in heat-shock response and proteostasis renders it a crucial drug target, which remained untapped yet. Here, we report for the first time the inhibitory potential of four small non-peptidic chemical molecules including SHS-II-123a, SHS-II-147a, US-IV-89, and US-IV-92 against the HslV protease using *in-silico* and *in vitro* techniques.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper..

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Availability of Data

The data that supports the findings of this study is available upon reasonable request.

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Ethical Approval

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

Authors' Contribution

Mehwish Hamid is the principal author and conducted most of the experimental work. Sana Aurangzeb performed some of the experimental work and result analysis. Yasmeen Rashid contributed to the conception and design of this study, data analysis and manuscript editing. Shahbaz Shamim and Uzma Salar provided purified triazines and hydrazinyl thiazole substituted chromones compounds, respectively. M. Kamran Azim, Khalid Mohammed Khan and Shahid Bashir critically reviewed and revised the manuscript. All authors have read and approved the final manuscript.

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