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Fluorometric CCHFV OTU protease assay with potent inhibitors

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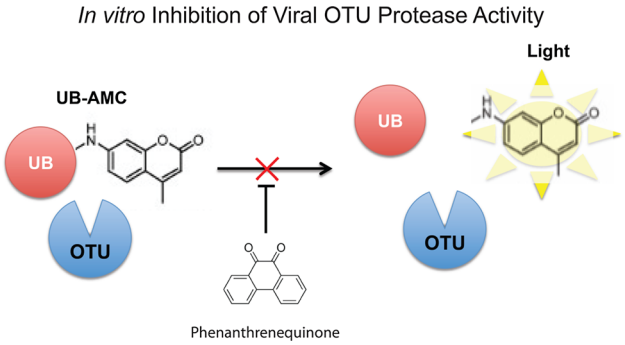
Abstract Crimean-Congo hemorrhagic fever virus (CCHFV) is a deadly virus that has been listed in the Category C as a potential bioterror agent. There are no specific therapies against CCHFV, which urges identification of potential therapeutic targets and development of CCHFV therapies. CCHFV OTU protease takes an important role in viral invasion through antagonizing NF-κB signaling. Inhibition of CCHFV OTU protease by small molecules warrants an exciting potential as antiviral therapeutics. Here we report the expression and purification of a C-His-tagged recombinant CCHFV OTU protease in *E. coli* BL21 (DE3) host strain. Activity of the refolded purified recombinant viral OTU protease has been validated with a UB-AMC fluorescent assay. In addition, we show a dose-dependent inhibition of the viral OTU protease by two small molecules. This study provides a reliable approach for recombinant expression and purification of CCHFV OTU protease, and demonstrates validation of OTU protease activity and its inhibition based on a UB-AMC florescent assay.

Graphical Abstract



Keywords Viral OTU protease · Expression and purification · Fluorescent assay · Antivirals · Crimean-Congo hemorrhagic fever virus · CCHFV · CCHF

Introduction

Crimean-Congo Hemorrhagic Fever Virus (CCHFV) is a deadly tick-borne virus belonging to the genus *Nairovirus* in the family of *Bunyaviridae*. The tick-borne properties of the nairoviruses, and the large L segment existing in their genome make them different from other members of the *Bunyaviridae* viruses [1, 2]. Crimean-Congo Hemorrhagic Fever (CCHF) constitutes a public health treat due to its high mortality rate in hospitalized patients [1–10]. It has been first reported in both the Crimea and Congo, since then there have been outbreaks in different parts of the world including Africa, Asia, and Eastern Europe [5, 9, 11–14]. Due to the potential travel of CCHFV-infected ticks with migratory birds, CCHF raises a concern regarding general healthcare in the world [15–17].

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The diagnosis of CCHF mainly depends on a high fever after a tick bite. The laboratory test for measurement of thrombocytopenia, leukopenia, and a high level of serum AST and ALT confirms the infection. Due to requirements of Biosafety Level 4, other molecular methods to diagnose CCHFV cannot be performed in many countries. However, a reverse transcription polymerase chain reaction method has been used as a standard molecular method to diagnose of CCHFV [1]. Unfortunately, current treatments based on ribavirin are not sufficient to treat CCHF disease and there is currently no commercial vaccine available for CCHFV. CCHFV Conflict of interesthas high viraemia, which is partly associated with its invasion mechanism that shuts down the antiviral response pathways with CCHFV ovarian tumor (OTU) protease. CCHFV OTU protease demonstrates broad deconjugation activities including deubiquitination [1, 18, 19].

The CCHFV OTU domain is part of the L segment. It takes a role in invasion of cells through antagonizing NF-κB signaling [1, 20]. Viral OTU differs from mammalian counterparts by its broad deubiquitination and removal of ISG15 modification in the infected cells [1, 3, 5, 7, 9, 10]. The crystal structure of viral OTU protease has been resolved along with ubiquitin (UB) and ISG15 proteins providing a structural approach to determine a pharmaceutical target site [2, 4–6, 8, 21, 22]. In addition, we have recently determined the inhibition pocket of CCHFV OTU protease (unpublished data). Moreover, mutation studies in several amino acids in close proximity to this pocket and the interaction of UB and ISG15 with the viral OTU protein as determined by crystallography studies provided further proof that the this inhibition pocket could be a pharmaceutical target for CCHFV OTU inhibitors [1, 11–14]. These studies provided us tools to uncover potent inhibitors of CCHFV OTU using computational chemistry.

There is an urgent need to develop small molecule inhibitors of CCHFV OTU. A couple of preliminary studies reported in the PubChem database provide compounds having potential inhibition of OTU protease. However, a reliable approach to determine antivirals targeting CCHFV OTU protease was needed. This study reports the expression and purification of a C-His-tagged recombinant CCHFV OTU protease in bacteria, followed by purification and validation of OTU protease activity using a UB-AMC fluorescent assay. In addition, we show the inhibition of CCHFV OTU protease by two potent inhibitors.

Materials and methods

Materials

E. coli DH5α was used as the host-vector system for miniprep and midiprep studies. *E. coli* BL21 (DE3) and Origami™ (DE3) strains were from Novagen and were used as the hosts for protein expression. pET-26b(+) plasmid was used to construct the expression vector. The restriction enzymes *Nco*I and *Nde*I were obtained from NEB Biosciences. The In-Fusion Cloning system, which also includes gel purification system and Stellar Competent cells, was purchased from Clontech. Primers and gene fragments used in this study were purchased from Integrated DNA Technologies (IDT). HisTrap HP (17-5248-01) was purchased from GE Healthcare and is used for Ni-Based column purification of His-Tagged protein. Ubiquitin-AMC (U-550) and AMC (B-200) were purchased from Boston Biochem, USA. Phenanthrenequinone was purchased from Sigma-Aldrich (275034). Homidium bromide was purchased from Invitrogen, USA.

Construction of bacterial expression vector

Recombinant CCHFV OTU protein was codon optimized for expression in *E. Coli*_K12 using OPTIMIZER (http://genomes.urv.es/OPTIMIZER/) [1, 20, 23]. The nucleotide sequence of the CCHFV OTU domain in this study is from a Turkey strain of CCHFV L segment (GenBank: DQ211623.1). Codon-optimized Recombinant OTU nucleotide sequence with C terminus His Tag was synthesized as two gBlocks® Gene Fragments (IDT, USA) and cloned into pET-26b(+) Vector (Cat#69862-3, Novagen) using the In-Fusion® HD cloning system (Cat#639645, Clontech) according to the manufacturer's recommendations. Shortly, we have linearized the pET26b(+) vector with *Nco*I and *Nde*I restriction enzymes (NEB England Biolabs) overnight and followed by gel purification provided in the In-Fusion® HD cloning system. We obtained two gBlocks from IDT (USA), which had homology sites for both ends (Supplementary Fig. 1). IDT geneblock stocks were 200 ng total, which were dissolved in 20 μL of TE buffer (10 mM Tris, 0.1 mM EDTA, pH 7.5). 2 μL of In-Fusion Enzyme Premix, 2 μL Linearized Vector (50–200 ng), 2.5 μL gene fragment 1, and 2.5 μL gene fragment 2 were mixed and incubated at 50 °C for 15 min, and then placed on ice. 5 μL of In-Fusion cloning reaction was transformed into Stellar competent *E. Coli* cells provided in the In-Fusion® HD cloning system and later colonies were screened with *Nde*I and *Nco*I digestion. pET-26b-CCHFV OTU vector sequence was verified by following primers: pET26b-F-seq 5′-AGATCTCGATCCCGCGAAAT-3′ and pET26b-R-seq 5′-GCCAACTCAGCTTCCTTTCG-3′.

Recombinant viral OTU protease expression in *E. coli*

BL21 (DE) and Origami™ (DE3) competent cells were used for testing expression of the OTU protein. Following

transformation of pET-26b-CCHFV OTU and induction at OD = 0.6 with 1 mM IPTG, samples were incubated at 25 and 37 °C overnight and OTU expression was studied by SDS-Page the following day. Western blot analysis using an Anti-His Tag antibody (Pierce) was used to confirm the expression of the OTU protease.

Purification of viral OTU protease

We used BL21 (DE) strain of *E. coli* due to its high growth kinetics for large-scale viral OTU protease purification. Shortly, bacterial samples following IPTG induction at 25 °C were lysed in solubilization buffer (8 M Urea, 20 mM *Tris*–HCL pH 8.0) by sonication for 5 min and cleared by spinning down to remove the debris and followed by filtering the supernatant with 0.45 μm filters. His-Tagged Recombinant CCHFV OTU protease was purified using HisTrap HP Columns (1 mL GE Healthcare) following the manufacturer's recommendations using the ÄKTA pure protein purification system. We have used buffer containing 8 M Urea, 500 mM NaCl, 50 mM Phosphate Buffer, 20 mM Imidazole, 14.1 μM 2-Mercaptoethanol as Wash/Equilibrium/Binding Buffer, and buffer containing 8 M Urea 500 mM NaCl, 50 mM Phosphate Buffer, 500 mM Imidazole, 14.1 μM 2-Mercaptoethanol as elution buffer. We collected samples at various stages of the purification and run on the SDS-PAGE to determine the purity of the purified protein. BSA was used as a molecular weight marker to estimate the amount of the loaded protein. We later exchanged the buffer of purified viral OTU protein with PBS using spin desalting columns and stored in PBS buffer at −20 °C.

Mass spec analysis

HisTrap HP Column-purified OTU samples were run on an SDS-PAGE gel, and the corresponding band of protein was excised and submitted to the Mass Spec facility, Houston for analysis.

Binding affinity calculation

3D SDF files of homidium (CID_3624) and phenanthrenequinone (CID_6763) used in this study were downloaded from the PubChem database [15–17, 24, 25]. Docking studies were performed using AutoDock Vina 1.1.2 [1, 18, 19, 26] and automated using PaDEL-ADV [1, 20, 27, 28]. Docking and binding affinity calculations were performed with a 22-20-20 Å search space centered on the pocket of the UB-OTU interaction. OTU-ligand diagrams were prepared using the AutoDockTools program (MGL Tools, The Scripps Research Institute).

CCHFV OTU Protease	Viral OTU domain of CCHFV L segment with C terminus 6X His Tag
Aminoacids	197 aa
Molecular Mass	21.7 kDa
Theoretical pI (ExPASy pI)	5.34
Signal Peptide	No
Transmembrane domain	No
GPI anchor (GPI Lipid Anchor Project)	No
Predicted Glycosylation sites (GlycoPred)	5N, 9T, 23N, 51N, 56S, 84S, 101T, 114T, 119T, 139T, 169S, 181T, 182S, 184S
Predicted disulfide bonds (DiANNA 1.1)	No (Number of Cysteine: 1)

Fig. 1 Viral OTU protease protein overview

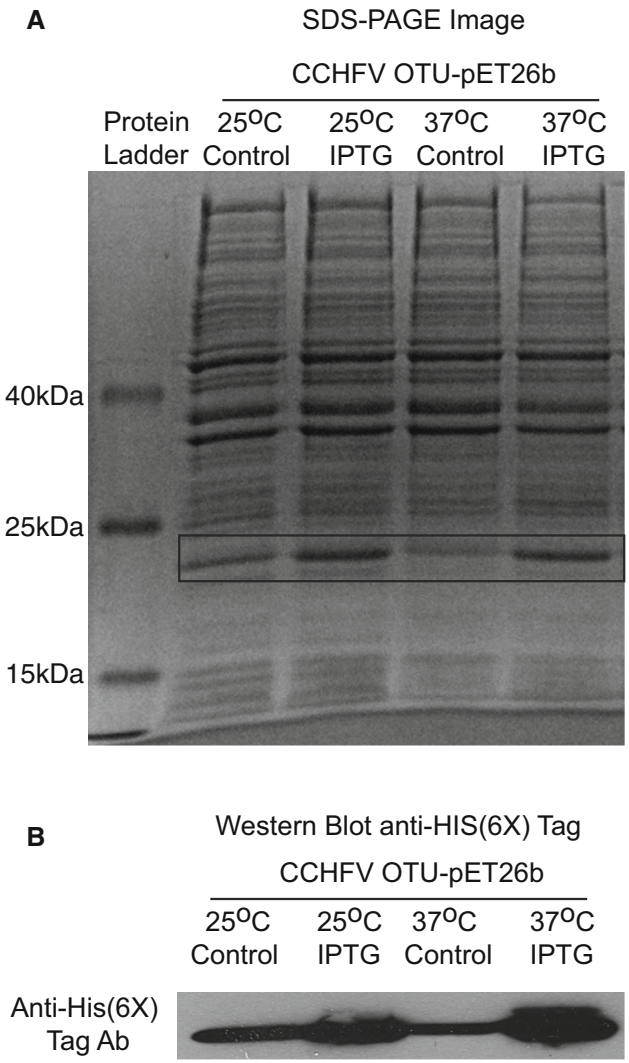

Fig. 2 Expression of recombinant CCHFV OTU protease. **a** Following transformation into BL21 (DE) cells, protein expression is induced via introduction of 1 mM IPTG at 25 and 37 °C. SDS-PAGE shows induction of CCHFV OTU at various temperatures. Expected size of His-Tagged viral OTU protein is 21.7 kDa. **b** Western blot analysis of induced (1 mM IPTG) and uninduced control samples (with some leaky expression) confirms expression of recombinant His-Tagged OTU protein

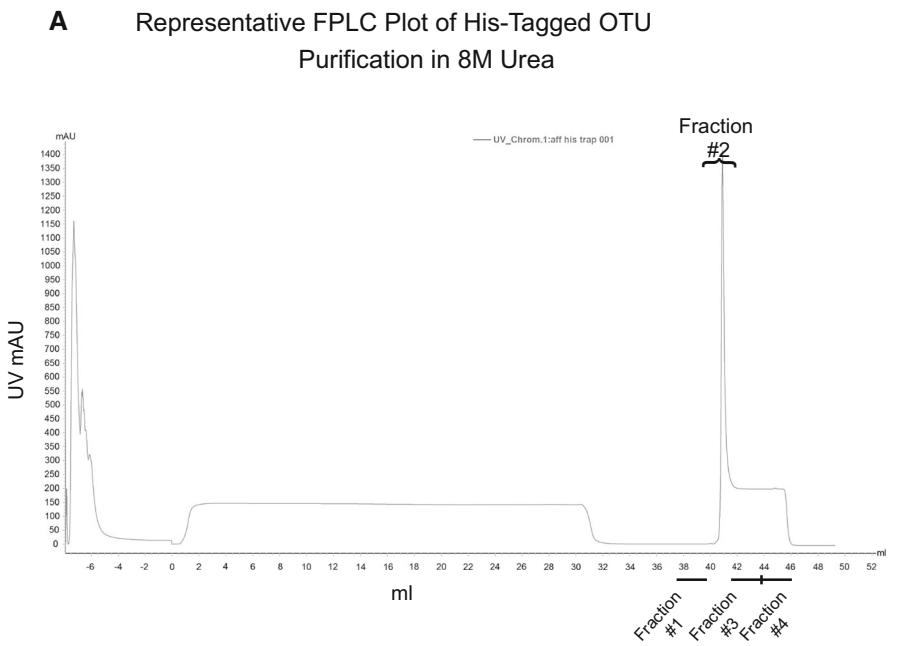

Fig. 3 Purification of C-His-Tagged OTU. **a** Representative FPLC plot of His-Tagged OTU protein purification method as performed in ÄKTA Pure with HisTrap HP (1 mL) column. **b** Purity analysis of recombinant OTU protein following FPLC purification by SDS-PAGE. Note that purity of OTU protein is >95 %

Fluorometric assay

Protease activity of the recombinant CCHFV OTU domain was tested using a UB-aminomethylcoumarin (AMC) system (U-550 and B-200, Boston Biochem, USA). Varying concentrations of viral OTU protease (6.25–400 nM) were incubated with different UB-AMC concentrations (100, 10, 1 nM) at 25 °C incubator. Buffer containing 10 mM HEPES, 100 mM NaCl, and 2.5 mM DTT (pH 7.5) was used as the reaction buffer as described previously with modifications [5]. Viral OTU protease activity (AMC fluorescence) was measured using a Synergy Neo HTS Multi-Mode Microplate Reader (Biotek) at an excitation of 360 nm and emission at 460 nm. Inhibition of CCHFV OTU by homidium (CID_3624) and phenanthrenequinone (CID_6763) (275034, Sigma-Aldrich, USA) was tested at the indicated concentrations (Fig. 8). Instead of homidium, we used homidium bromide (Invitrogen, USA).

Statistical analysis

Statistical analyses were performed using the Student's t test. p values < 0.05 were considered statistically significant.

MDFLRNLDWTQVIAGQYVTNPRFNISDYFEIVRQPGDGNCFYHSIAELTMPNKTAHSYHNIKHLTE

LAARKYYQEEPEAKLVGLSLEDYLKRMLSDNEWGSTLEASMLAKEMGITIIIWTVAASDEVEAGIK

FGDGDVFTAVNLLHSGQTHFDALRILPQFETDTREALSLVDKVMAVDQLTSSSHHHHHH*

Fig. 4 Mass spec analysis of recombinant CCHFV OTU protease. Verified amino acids are shown in orange and underlined

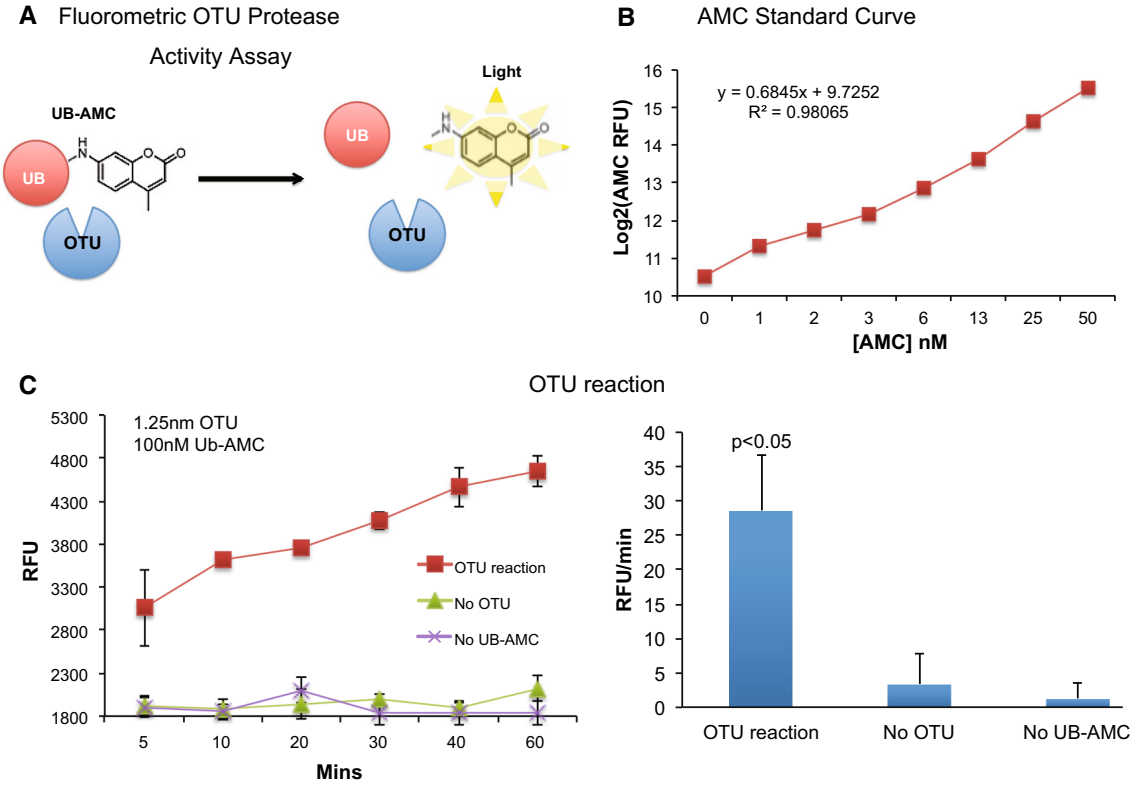

Fig. 5 In vitro UB-deconjugation activities of Viral OTU Protein. **a** schematic of fluorometric OTU protease activity assay. **b** AMC standard curve of fluorometric assay. **c** *Left* Time-dependent activity of viral OTU protease with 100 nM UB-AMC substrate and 1.25 nM viral OTU protease. *Right* Activity of viral OTU protease with 100 nM UB-AMC substrate and 1.25 nM viral OTU protease in vitro post 60 min versus $t = 0$. Note that OTU Fluorometric assay setup shows high levels of signal to noise ratio even at very low viral OTU protease concentration. $p < 0.05$, $n = 3$

Resultss

Expression and purification of recombinant CCHFV OTU protease

We have cloned recombinant CCHFV OTU protease into a bacterial expression plasmid to study its in vitro activities and the inhibitory efficacy of two small molecules. In order to achieve a high degree of expression in bacterial settings, the OTU nucleotide sequence has been optimized to bacterial codon preference using OPTIMIZER (Supplementary Table 1). We have included His Tag at the C terminus of optimized recombinant OTU Protease (Fig. 1) and cloned into pET-26b vector using the In-Fusion cloning system with two fragments containing homology sequences (Supplementary Fig. 1). We have tested pET-26b-OTU plasmid in Origami and BL21 (DE) strains of bacteria. Analysis of different temperatures showed that recombinant CCHFV OTU protease could be expressed both at 25 and 37 °C following 1 mM IPTG induction (Fig. 2a). Expression of OTU protein is also confirmed by western blotting using anti-his antibody (Fig. 2b). We found that BL21 (DE) strain provided better growth kinetics and expression levels. Thus, BL21 was used for the rest of the OTU expression and purification studies. Using HisTrap HP columns, we performed CCHFV OTU protease purification in an ÄKTA Pure FPLC system (Fig. 3a). Samples from the elution step were divided into fractions. Fraction 2 provided the highest levels of purified CCHFV

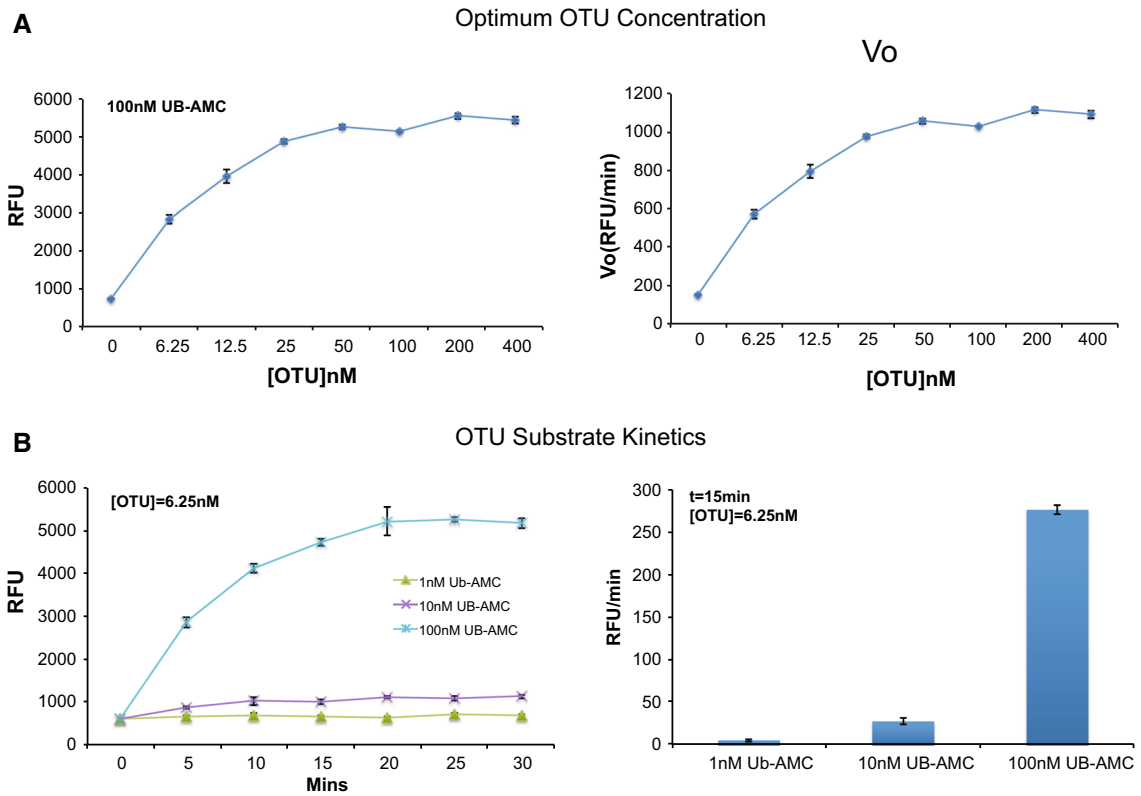

Fig. 6 Optimum viral OTU protease and substrate content. **a** Optimum OTU concentration and Vo is determined at constant UB-AMC substrate (0.1 μM) at ambient temperature and various OTU concentrations. **b** Substrate kinetics are determined at constant CCHFV OTU amount (6.25 nM) with varying UB-AMC concentrations (100, 10, and 1 nM) with indicated time points. $n = 3$

OTU protease (Fig. 3b). Moreover, we have confirmed the OTU protein sequence by Mass Spec analysis of purified OTU samples (Fig. 4).

In vitro UB-deconjugation activities of recombinant CCHFV OTU protein

We have used a fluorometric OTU protease activity assay to study activity of purified recombinant OTU protease (Fig. 5a). This assay uses UB protein attached with a fluorophore known as AMC, which is fluorescent only when it is cleaved from UB. Figure 5b shows the standard curve using the AMC fluorophore. When we incubate CCHFV OTU protease at 80 nM concentration along with 100 nM UB-AMC, we found that CCHFV OTU was functionally active and thus deconjugates UB-AMC (Fig. 5c). In addition, it was enough to cleave 100 nM UB-AMC with as low as 6.25 nM CCHFV OTU protease at 25° (Fig. 6a). Moreover, we have determined the CCHFV OTU protease substrate kinetics using varying UB-AMC concentrations ranging from 1, 10 and 100 nM (Fig. 6b). Further studies were carried out using 6.25 nM OTU and 100 nM UB-AMC to get the best signals during inhibition assays.

In vitro inhibition of CCHFV OTU protease

We have performed molecular docking using Autodock Vina and CCHFV OTU protease inhibition assays of readily available compounds such as CID_3624 (homidium), and CID_6763 (Phenanthrenequinone). We have determined that homidium and phenanthrenequinone preferentially bind to the pocket of the OTU–UB interaction with predicted binding affinities of −6.5 and −6.3 kcal/mol, respectively (Fig. 7). In vitro inhibition assays on CCHFV OTU protease with CID_77186, and CID_3624 demonstrates a dose-dependent inhibition with homidium and phenanthrenequinone (Fig. 8 and supplementary Fig. 2). Ribavirin, a known antiviral that specifically targets RNA-dependent RNA polymerase, is used as a negative control in CCHFV fluorometric protease assays and does not inhibit the viral OTU protease.

Discussion

This study provides a cost-effective and time-saving process for expression, purification, and assessment of inhibitors of CCHFV OTU protease. In addition, we have

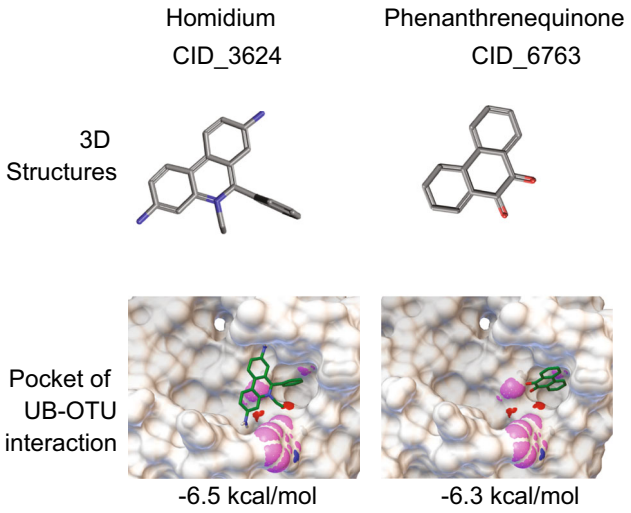

Fig. 7 Molecular docking of small molecules OTU protease activity pocket. We have performed a virtual screen and found that homidium (CID_3624) and phenanthrenequinone (CID_6763) have predicted binding affinity of −6.5 and −6.3 kcal/mol towards protease activity pocket, respectively. *Top* 3D structures of the small molecules. *Bottom* Maximum binding affinity poses of the small molecules in viral OTU protease activity pocket

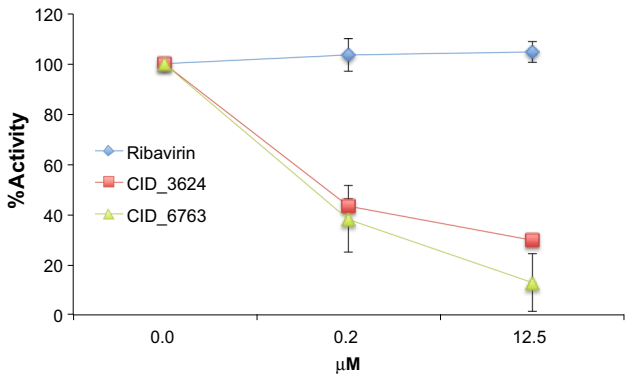

Fig. 8 In vitro inhibition of CCHFV OTU protease activity. Homidium (CID_3624) and phenanthrenequinone (CID_6763) show dose-dependent inhibition of CCHFV OTU Protease. Ribavirin is used as negative control in CCHFV OTU fluorometric protease assay. $n = 3$

tested two potential viral OTU inhibitors that could be used in further evaluations for treatment of CCHFV infections. Especially, inhibition of viral OTU protease in vitro by the compound phenanthrenequinone demonstrates the possibility of CCHFV specific therapeutics.

Phenanthrenequinone, also known as 9,10-phenanthrenequinone, leads to increased proliferation of human A549 cells at 5 μM concentrations without any obvious cell death, and this is largely found to be associated with a burst of reactive oxygen species (ROS) [29]. Increased ROS levels are largely associated with the AKR1B10 enzyme involved in the phenanthrenequinone redox-cycle, thereby causing ROS production [29]. On the other hand, phenanthrenequinone could cause apoptosis when cells are exposed to high levels (>50 μM). We have shown that 50 % inhibition of viral OTU by phenanthrenequinone occurs at concentrations lower than 0.2 μM (Fig. 8). Viral OTU is part of a group of proteases with deubiquitinase function. Studies indicate that increased levels of ROS could inactivate deubiquitinases through oxidation of a catalytic cysteine residue [30]. Thus, treatment of CCHFV-infected cells with phenanthrenequinone could provide both inhibition of viral OTU through contact inhibition as well as oxidation of catalytic cysteines through a burst of ROS.

Bergeron et al. showed that deletion of viral OTU protease in L segment of CCHFV still results in a comparable RdRp activity indicating that viral OTU protease does not significantly regulate RdRp activity [31]. Viral OTU protease is located in the L segment of CCHFV genome and provides an invasive advantage through antagonizing NF-κB pathway, which prevents the innate immune system response. In addition, viral OTU domain in nairobi sheep disease virus RdRp L segment shows an antagonistic effect on the induction of interferon proteins and innate immune system through reduction of host protein ubiquitinations [7]. Accumulating evidence indicated that viral OTU protease involves in the interference of host innate immune system; thus, inhibition strategies for viral OTU protease could be used against CCHFV infections. Thus, fluorescent CCHFV OTU protease assay provides a basis for the screening of CCHFV OTU inhibitors that might have antiviral activity against CCHFV. Furthermore, small molecule inhibitors of viral OTU protease may serve not only for the development of treatments for CCHFV infection but also could be applied to other CCHFV-related viruses and intracellular parasites that uses homologs of OTU protease in cellular invasion. This study could be extended to evaluate biological activity of identified compounds in BSL4 facilities on CCHFV itself and animal models of CCHF disease that would help in designing compounds with higher potency and lower toxicity and development of CCHF therapeutics.

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Compliance with Ethical Standards

Conflict of interest All authors declare that they have no conflicts of interest concerning this work.

References

1. N. Frias-Staheli, N.V. Giannakopoulos, M. Kikkert, S.L. Taylor, A. Bridgen, J. Paragas, J.A. Richt, R.R. Rowland, C.S. Schmaljohn, D.J. Lenschow, E.J. Snijder, A. García-Sastre, H.W. Virgin, Ovarian tumor domain-containing viral proteases evade ubiquitin- and ISG15-dependent innate immune responses. Cell Host Microbe **2**(6), 404–416 (2007)
2. D.A. Bente, N.L. Forrester, D.M. Watts, A.J. McAuley, C.A. Whitehouse, M. Bray, Crimean-Congo hemorrhagic fever: history, epidemiology, pathogenesis, clinical syndrome and genetic diversity. Antiviral Res. **100**, 159–189 (2013)
3. G.C. Capodagli, M.K. Deaton, E.A. Baker, R.J. Lumpkin, S.D. Pegan, Diversity of ubiquitin and ISG15 specificity among nairoviruses' viral ovarian tumor domain proteases. J. Virol. **87**, 3815–3827 (2013)
4. K. Ergunay, C.A. Whitehouse, A. Ozkul, Current status of human arboviral diseases in Turkey. Vector Borne Zoonotic Dis. **11**, 731–741 (2011)
5. G.C. Capodagli, M.A. McKercher, E.A. Baker, E.M. Masters, J.S. Brunzelle, S.D. Pegan, Structural analysis of a viral ovarian tumor domain protease from the crimean-congo hemorrhagic fever virus in complex with covalently bonded ubiquitin. J. Virol. **85**, 3621–3630 (2011)
6. E. Ozkaya, E. Dincer, A. Carhan, Y. Uyar, M. Ertek, C.A. Whitehouse, A. Ozkul, Molecular epidemiology of Crimean-Congo hemorrhagic fever virus in Turkey: occurrence of local topotype. Virus Res. **149**, 64–70 (2010)
7. B. Holzer, S. Bakshi, A. Bridgen, M.D. Baron, Inhibition of interferon induction and action by the nairovirus Nairobi sheep disease virus/Ganjam virus. PLoS One **6**(12), e28594 (2011)
8. C.A. Whitehouse, Crimean-Congo hemorrhagic fever. Antiviral Res. **64**, 145–160 (2004)
9. M. Akutsu, Y. Ye, S. Virdee, J.W. Chin, D. Komander, Molecular basis for ubiquitin and ISG15 cross-reactivity in viral ovarian tumor domains. Proc. Natl. Acad. Sci. **108**, 2228–2233 (2011)
10. O.A. Malakhova, D.-E. Zhang, ISG15 inhibits Nedd4 ubiquitin E3 activity and enhances the innate antiviral response. J. Biol. Chem. **283**, 8783–8787 (2008)
11. O. Ergonul, Crimean-Congo hemorrhagic fever virus: new outbreaks, new discoveries. Curr. Opin. Virol. **2**, 215–220 (2012)
12. A. Gargili, K. Midilli, O. Ergonul, S. Ergin, H.G. Alp, Z. Vatansever, S. Iyisan, C. Cerit, G. Yilmaz, K. Altas, A. Estrada-Peña, Crimean-Congo hemorrhagic fever in European part of Turkey: genetic analysis of the virus strains from ticks and a seroepidemiological study in humans. Vector Borne Zoonotic Dis. **11**, 747–752 (2011)
13. B. Dokuzoguz, A.K. Celikbas, S.E. Gök, N. Baykam, M.N. Eroglu, O. Ergonul, Severity scoring index for crimean-congo hemorrhagic Fever and the impact of ribavirin and corticosteroids on fatality. Clin. Infect. Dis. **57**, 1270–1274 (2013)
14. O. Ergonul, A.K. Celikbas, B. Dokuzoguz, S.E. Gök, N. Baykam, H. Esener, Characteristics of patients with Crimean-Congo hemorrhagic fever in a recent outbreak in Turkey and impact of oral ribavirin therapy. Clin. Infect. Dis. **39**(2), 284–287 (2004)
15. M. Lindeborg, C. Barboutis, C. Ehrenborg, T. Fransson, T.G.T. Jaenson, P.-E. Lindgren, A. Lundkvist, F. Nyström, E. Salaneck, J. Waldenström, B. Olsen, Migratory birds, ticks, and Crimean-Congo hemorrhagic fever virus. Emerg. Infect. Dis. **18**, 2095–2097 (2012)
16. A.M. Palomar, A. Portillo, P. Santibáñez, D. Mazuelas, J. Arizaga, A. Crespo, Ó. Gutiérrez, J.F. Cuadrado, J.A. Oteo, Crimean-Congo hemorrhagic fever virus in ticks from migratory birds. Morocco. Emerg. Infect. Dis. **19**, 260–263 (2013)
17. P. Gale, B. Stephenson, A. Brouwer, M. Martinez, A. la de Torre, J. Bosch, M. Foley-Fisher, P. Bonilauri, A. Lindström, R.G. Ulrich, J. de Vos, M. Scremin, L. Kelly, Z. Liu, M.J. Muñoz, Impact of climate change on risk of incursion of Crimean-Congo haemorrhagic fever virus in livestock in Europe through migratory birds. J. Appl. Microbiol. **112**, 246–257 (2012)
18. F. Weber, A. Mirazimi, Interferon and cytokine responses to Crimean Congo hemorrhagic fever virus; an emerging and neglected viral zonoosis. Cytokine Growth Factor Rev. **19**, 395–404 (2008)
19. M.D. Arguello, J. Hiscott, Ub surprised: viral ovarian tumor domain proteases remove ubiquitin and ISG15 conjugates. Cell Host Microbe **2**, 367–369 (2007)
20. P.B. van Kasteren, C. Beugeling, D.K. Ninaber, Arterivirus and nairovirus ovarian tumor domain-containing deubiquitinases target activated RIG-I to control innate immune signaling. J. Virol. **86**(2), 773–785 (2012)
21. T.W. James, N. Frias-Staheli, J.-P. Bacik, J.M. Levingston Macleod, M. Khajehpour, A. García-Sastre, B.L. Mark, Structural basis for the removal of ubiquitin and interferon-stimulated gene 15 by a viral ovarian tumor domain-containing protease. Proc. Natl. Acad. Sci. **108**, 2222–2227 (2011)
22. E. Kinsella, S.G. Martin, A. Grolla, M. Czub, H. Feldmann, R. Flick, Sequence determination of the Crimean-Congo hemorrhagic fever virus L segment. Virology **321**(1), 23–28 (2004)
23. P. Puigbò, E. Guzmán, A. Romeu, S. Garcia-Vallvé, OPTIMIZER: a web server for optimizing the codon usage of DNA sequences. Nucleic Acids Res. **35**, W126–W131 (2007)
24. E.E. Bolton, Y. Wang, P.A. Thiessen, S.H. Bryant, PubChem: integrated platform of small molecules and biological activities. Annu. Rep. Comput. Chem. **4**, 217–241 (2008)
25. Y. Wang, J. Xiao, T.O. Suzek, J. Zhang, J. Wang, Z. Zhou, L. Han, K. Karapetyan, S. Dracheva, B.A. Shoemaker, E. Bolton, A. Gindulyte, S.H. Bryant, PubChem's bioassay database. Nucleic Acids Res. **40**, D400–D412 (2011)
26. O. Trott, A.J. Olson, AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. J. Comput. Chem. **31**(2), 455–461 (2009)
27. M.F. Sanner, A.J. Olson, J.C. Spehner, Reduced surface: an efficient way to compute molecular surfaces. Biopolymers **38**, 305–320 (1996)
28. M.F. Sanner, Python: a programming language for software integration and development. J. Mol. Graph. Model. **17**, 57–61 (1999)
29. T. Matsunaga, Y. Morikawa, M. Haga, S. Endo, M. Soda, K. Yamamura, O. El-Kabbani, K. Tajima, A. Ikari, A. Hara, Exposure to 9,10-phenanthrenequinone accelerates malignant progression of lung cancer cells through up-regulation of aldo-keto reductase 1B10. Toxicol. Appl. Pharmacol. **278**, 180–189 (2014)
30. X.M. Cotto-Rios, M. Békés, J. Chapman, B. Ueberheide, T.T. Huang, Deubiquitinases as a signaling target of oxidative stress. Cell Rep. **2**, 1475–1484 (2012)
31. E. Bergeron, C.G. Albarino, M.L. Khristova, S.T. Nichol, Crimean-Congo hemorrhagic fever virus-encoded ovarian tumor protease activity is dispensable for virus RNA polymerase function. J. Virol. **84**, 216–226 (2010)