Flipping the substrate preference of Hazara virus ovarian tumour domain protease through structure-based mutagenesis

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Nairoviruses are arthropod-borne viruses with a nearly global geographical distribution. Several are known causative agents of human disease, including Crimean–Congo hemorrhagic fever virus (CCHFV), which has a case fatality rate that can exceed 30%. Nairoviruses encode an ovarian tumour domain protease (OTU) that can suppress the innate immune response by reversing post-translational modifications by ubiquitin (Ub) and/or interferon-stimulated gene product 15 (ISG15). As a result, the OTU has been identified as a potential target for the development of CCHFV therapeutics. Despite sharing the same general fold, nairoviral OTUs show structural and enzymatic diversity. The CCHFV OTU, for example, possesses activity towards both Ub and ISG15, while the Hazara virus (HAZV) OTU interacts exclusively with Ub. Virology studies focused on the OTU have mostly been restricted to CCHFV, which requires BSL-4 containment facilities. Although HAZV has been proposed as a BSL-2 alternative, differences in the engagement of substrates by CCHFV and HAZV OTUs may present complicating factors when trying to model one using the other. To understand the molecular underpinnings of the differences in activity, a 2.78 Å resolution crystal structure of HAZV OTU bound to Ub was solved. Using structure-guided site-directed mutagenesis, HAZV OTUs were engineered with altered or eliminated deubiquitinase activity, including one with an exclusive activity for ISG15. Additionally, analysis of the structure yielded insights into the difference in inhibition observed between CCHFV and HAZV OTUs with a Ub-based inhibitor. These new insights present opportunities to utilize HAZV as a model system to better understand the role of the OTU in the context of infection.

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1. Introduction

Crimean–Congo hemorrhagic fever virus (CCHFV) is a negative-sense, single-stranded RNA virus in the *Nairoviridae* family. Nairoviruses are tick-borne viruses within the *Bunyavirales* order, representing ~40 of the more than 350 viruses within this taxon (Kuhn *et al.*, 2016; Maes *et al.*, 2018, 2019; Walker *et al.*, 2016). Of the nairoviruses, CCHFV has the greatest impact on human health. CCHFV causes mild to severe disease, with reported fatality rates that can exceed 30%, and is endemic to Africa, the Middle East, Asia, and portions of eastern and southern Europe, making it one of the most widespread hemorrhagic fever-causing viruses (Bente *et al.*, 2013). In addition to tick-borne infections, nosocomial transmission of CCHFV has also been a significant factor in outbreaks (Tsergouli *et al.*, 2020). It has also shown a propensity to spread to new regions, highlighted by the

emergence of CCHFV in Spain in both humans and ticks (Negredo *et al.*, 2017). Owing to the concerns of the severity and spread of the disease and the lack of specific therapeutics, the World Health Organization has identified CCHFV as a high-priority pathogen for which there is an urgent need for accelerated research and development (Friedrich, 2018).

As part of its multifunctional L protein, CCHFV encodes an ovarian tumour domain protease (OTU) that possesses deubiquitinase (DUB) and deISGylase activities (Bergeron *et al.*, 2010; Frias-Staheli *et al.*, 2007). The activity of the OTU in reversing post-translational modifications of proteins by ubiquitin (Ub) and interferon-stimulated gene product 15 (ISG15) has been shown to suppress the innate immune response of host cells to CCHFV and other nairoviruses (Bakshi *et al.*, 2013; Frias-Staheli *et al.*, 2007; Holzer *et al.*, 2011; Scholte *et al.*, 2017; van Kasteren *et al.*, 2012). Owing to this, the OTU is considered to be a potential therapeutic target. Using a phage-display library, Zhang and coworkers developed a Ub variant (UbV) with high affinity for the OTU and inhibition of its activity (Zhang *et al.*, 2017). Recently, this inhibitor (UbV-CC4) has been confirmed to inhibit CCHFV replication in cell culture (Scholte *et al.*, 2019). Interestingly, this inhibition occurred independently of the interferon-suppressive function of the OTU, indicating a potentially greater role of the OTU that presents opportunities for pursuing it as a therapeutic target.

Owing to the severity of the disease, research with authentic CCHFV is restricted to high-containment facilities. As a result, other nairoviruses, such as Hazara virus (HAZV), have been proposed as alternative models that can be studied at the BSL-2 level (Dowall *et al.*, 2012; Flusin *et al.*, 2011; Matsumoto *et al.*, 2018; Molinas *et al.*, 2016). HAZV causes a fatal disease in immunocompromised mice that largely resembles CCHFV (Dowall *et al.*, 2012; Zivcec *et al.*, 2013). However, the differences between CCHFV and HAZV may create some limitations in extending results from these systems to practical applications. The OTUs, for example, possess different activity profiles towards Ub and ISG15, with CCHFV OTU interacting with both while HAZV OTU only engages Ub (Dzimianski, Beldon *et al.*, 2019; Dzimianski, Scholte *et al.*, 2019). As a result, it is not precisely known how the immune-suppression activities of these viruses differ. Additionally, the UbV-CC4 inhibitor is highly selective for CCHFV OTU, showing a 10-fold weaker inhibition of HAZV OTU (Scholte *et al.*, 2019). In order to properly relate findings for HAZV to CCHFV, a greater understanding is needed of their differences in order to properly design experimental systems of nairoviral disease.

To gain more insight into these differences, we solved a structure of HAZV OTU bound to Ub. This revealed that HAZV OTU binds to substrates in a distinct manner compared with other nairoviruses. Utilizing the structure as a guide, we constructed mutants that have variable enzymatic activities towards Ub and ISG15 substrates, including mutants with a completely reversed substrate preference. We further assessed the inhibition of HAZV OTU by UbV-CC4 to gain a greater understanding of the reduced inhibition relative to CCHFV.

2. Materials and methods

2.1. Expression and purification of HAZV OTU and Ub

HAZV OTU in pET-11a vector was expressed and purified as described previously (Capodagli *et al.*, 2011, 2013; Deaton *et al.*, 2016; Dzimianski, Beldon *et al.*, 2019; Dzimianski, Scholte *et al.*, 2019). Ub in pTYB2 vector was expressed, purified and derivatized with propargylamine to generate a suicide substrate (Ub-PA; Deaton *et al.*, 2016; Dzimianski, Scholte *et al.*, 2019; Wilkinson *et al.*, 2005). HAZV OTU was added to excess Ub-PA and incubated at room temperature for 2 h followed by overnight dialysis in buffer A (100 mM NaCl, 50 mM Tris pH 8.0) at 4°C. An Ni-NTA column (GoldBio) was used to remove excess Ub-PA from the sample. The column was washed with buffer A supplemented with 10 mM imidazole, and the protein was eluted with buffer A supplemented with 300 mM imidazole. The protein was dialyzed into buffer A overnight at 4°C and further purified by ion-exchange chromatography on a Mono Q 10/100 column. Following sample loading, the protein was eluted over a ten-column volume gradient of 0–50% buffer B (1 M NaCl, 50 mM Tris pH 8.0) followed by a three-column volume wash with 100% buffer B. The fractions were analysed based on the chromatogram and SDS-PAGE, and were then selected and pooled based on the presence and purity of the complex. The pooled fractions were concentrated and buffer-exchanged into buffer C (100 mM NaCl, 5 mM HEPES pH 7.5, 10 mM DTT) using a Superdex 75 column (GE Healthcare).

2.2. Crystallization of HAZV OTU–Ub

Fractions from size-exclusion chromatography containing the complex were pooled based on the chromatogram, confirmed by SDS-PAGE and concentrated to 10 mg ml⁻¹. The sample was screened in 96-well hanging-drop plates against a suite of 768 commercially available conditions (Qiagen) using a Mosquito robot (TTP Labtech). Rod-like crystals in different conditions formed in a time ranging from two weeks to six months. Hits from the screen were scaled up to hanging-drop 24-well plates containing a 500 µl reservoir of the crystallization solution and were optimized using varying salt, precipitant, pH, additive and protein concentration gradients. The final crystallization condition for the optimized crystals was 0.3 M CaCl₂, 20% PEG 3350.

2.3. Data collection and processing

Crystals were flash-cooled in liquid nitrogen using artificial mother liquor supplemented with 6% DMSO, 6% glycerol and 6% ethylene glycol as a cryoprotectant. Data were collected under a dry N₂ stream on the 22-BM beamline (SER-CAT) at Argonne National Laboratory using a MARmosaic CCD detector. The data were indexed, integrated and scaled in HKL-2000 (Otwinowski & Minor, 1997). Data-collection statistics are included in Table 1.

2.4. Structure solution and refinement

Phases for the HAZV OTU–Ub structure were solved by molecular replacement in *Phaser* (Adams *et al.*, 2010; McCoy *et al.*, 2007; Winn *et al.*, 2011) using a composite homology model generated in *MODELLER* (Šali & Blundell, 1993) using the CCHFV OTU–Ub (PDB entry 3prp), Dugbe virus (DUGV) OTU–Ub (PDB entry 4hxd), Erve virus (ERVEV) OTU–CmISG15 (PDB entry 5jze) and Kupe virus (KUPEV) OTU–CshISG15 (PDB entry 6oar) structures as templates (Capodagli *et al.*, 2011, 2013; Deaton *et al.*, 2016; Dzimianski, Scholte *et al.*, 2019). Firstly, the OTU alone was used as a search model, placing four copies in the asymmetric unit. This was followed by a search for Ub, placing one copy in the correct orientation compared with the OTU. This complete copy of the OTU–Ub complex was used as a guide to place the other copies of Ub. The complete initial model was rebuilt using the *AutoBuild* module in *Phenix*, followed by alternating rounds of manual building and refinement in *Coot* and *Phenix* (Emsley *et al.*, 2010; Liebschner *et al.*, 2019). The final model was validated in *MolProbity* (Chen *et al.*, 2010), and the associated refinement statistics are included in Table 1. The structure factors and final coordinates have been deposited in the Worldwide Protein Data Bank (PDB entry 7jms).

2.5. Site-directed mutagenesis of HAZV OTU

HAZV OTU mutants (S13I, S13E, A80I, L120T, L120V, G129S, G129A, L149N, L149R, L149Q, L149Y, A80I/G129S, A80I/G129S/L149Y, S13E/A80I/G129S and S13E/A80I/G129S/L149Y) were created using the QuikChange Lightning or QuikChange Lightning Multi site-directed mutagenesis kits according to the manufacturer's methods (Agilent Technologies). The mutant plasmids were transformed by heat shock into *Escherichia coli* NEB 5- α high-efficiency cells and then purified in preparation for sequencing. The mutations were confirmed by sequencing (Eton Bioscience) and the plasmids were transformed into *E. coli* T7 Express cells by heat shock for protein expression. The mutant proteins were expressed and purified as described previously (Capodagli *et al.*, 2011, 2013; Deaton *et al.*, 2016; Dzimianski, Beldon *et al.*, 2019; Dzimianski, Scholte *et al.*, 2019).

2.6. Enzymatic assays of HAZV OTU mutants

Assays were executed as described previously (Capodagli *et al.*, 2013; Deaton *et al.*, 2016; Dzimianski, Beldon *et al.*, 2019). Briefly, HAZV OTU mutant activity was evaluated against Ub-7-amido-4-methylcoumarin (Ub-AMC) and ISG15-AMC substrates (Boston Biochem, Massachusetts, USA). The final enzyme concentration in the assays was 4 nM for Ub-AMC and 20 nM for ISG15-AMC. Each substrate was present at a concentration of 1 μ M. The reactions were run in duplicate with 50 μ l volumes in 96-well Corning Costar half-volume plates in reaction buffer [100 mM NaCl, 50 mM HEPES pH 7.5, 0.01 mg μ l⁻¹ bovine serum albumin (BSA), 5 mM dithiothreitol (DTT)]. The reactions were initiated by the addition of OTU. Reaction rates were measured by the fluorescence generated from the cleavage of 7-amino-4-methylcoumarin

Table 1

Data-collection and refinement statistics.

Values in parentheses are for the highest resolution shell.

Data collection	
Space group	<i>P</i> 2 ₁
Wavelength (Å)	1
<i>a</i> , <i>b</i> , <i>c</i> (Å)	83.85, 55.60, 97.43
α , β , γ (°)	90, 97.20, 90
Resolution range (Å)	50–2.78 (2.83–2.78)
No. of unique reflections	22059 (2190)
Completeness (%)	96.9 (97.2)
Multiplicity	4.7
$\langle I/\sigma(I) \rangle$	10.8 (2.43)
<i>R</i> _{meas}	0.199 (0.781)
CC _{1/2}	0.99 (0.64)
Overall <i>B</i> factor from Wilson plot (Å ²)	47.17
Refinement	
Resolution range (Å)	40.61–2.78 (2.88–2.78)
Completeness (%)	96.9 (98.38)
Unique reflections	22052 (2190)
Final <i>R</i> _{work}	0.235 (0.305)
Final <i>R</i> _{free}	0.273 (0.340)
No. of non-H atoms	
Protein	7399
Ligands†	37
Water	270
R.m.s. deviations	
Bonds (Å)	0.006
Angles (°)	0.95
Average <i>B</i> factors (Å ²)	
Protein	43.39
Ligands	47.03
Water	41.26
Ramachandran plot	
Most favoured (%)	98.25
Allowed (%)	1.75

† Includes calcium, glycerol and the propargyl linker.

from the substrate using a CLARIOstar plate reader (BMG Labtech). The data were analysed using *MARS* (BMG Labtech). The initial reaction rates for each reaction were plotted and analysed based on steady-state kinetics.

2.7. Inhibition of HAZV OTU by UbV-CC4

Inhibition assays were run for the wild-type OTU with UbV-CC4 and UbV-CC4 mutants (generated as described above). For each reaction the protease, inhibitor and Ub-AMC substrate were combined. The final substrate concentration was 1 μ M, the final OTU concentration was 4 nM, the final inhibitor concentration was 1 μ M and the total reaction volume was 30 μ l. Each reaction was run in duplicate. The inhibitors and OTU were combined for 2 min prior to being added to the substrate to initiate the reaction.

3. Results and discussion

3.1. Structure of HAZV OTU–Ub

To generate a covalent complex, Ub was expressed, purified and converted into a propargylamine-based suicide substrate according to previously published methods (Deaton *et al.*, 2016; Dzimianski, Scholte *et al.*, 2019; Wilkinson *et al.*, 2005). This modified Ub was combined in excess with purified HAZV OTU, producing a complex with a covalent link between the catalytic cysteine of the OTU and the C-terminus of Ub.

Following additional purification to eliminate excess Ub and inactive OTU, the complex was screened against 768 unique conditions. This produced multiple hits that were subsequently optimized. Data from multiple isomorphous crystals were combined to yield a data set to 2.78 Å resolution in space group $P2_1$. An initial search model was generated in *MODELLER* based on multiple OTU–substrate complex structures from related viruses. The initial search successfully placed the OTU, with four copies in the asymmetric unit. A subsequent search for Ub successfully placed a copy that served as a guide for modelling the remaining three Ub chains.

Comparison with other nairoviral OTU–substrate complexes revealed some surprising differences in the way that HAZV OTU binds to Ub. Most of the nairoviral OTUs, including those from CCHFV, DUGV, ERVEV and KUPEV, demonstrate high similarity to each other in their overall mode of binding to Ub and/or ISG15 (Fig. 1). In contrast, HAZV OTU binds Ub in a noticeably different orientation. Compared with a CCHFV OTU–Ub structure, the Ub molecule in the HAZV OTU complex is tilted by 22.8° (Fig. 1*b*). Looking at the interface of the interaction reveals three points of difference in HAZV OTU versus other nairoviral OTUs that account for

Figure 1

Structural features of HAZV OTU bound to Ub. (a) Cartoon rendering of the HAZV OTU–Ub complex. The OTU is coloured cyan and Ub is coloured tan. The propargyl linker is shown in brown. Secondary structure is assigned based on the *DSSP* algorithm (Kabsch & Sander, 1983). (b) Overall comparison with other nairoviral OTU structures (CCHFV OTU–Ub, PDB entry 3ppr; DUGV OTU–Ub, PDB entry 4hxd; ERVEV OTU–C-mouse ISG15, PDB entry 5jze; GANV OTU–sheep ISG15, PDB entry 6oat; KUPEV OTU–C-sheep ISG15, PDB entry 6oar; FARV OTU, PDB entry 6dx5). The Ub molecule from the HAZV OTU complex was placed based on an overlay of the OTUs. The angles between substrate-binding orientations were calculated using the *anglebetweenhelices* plugin in *PyMOL* (version 2.3; Schrödinger). (c) Sequence alignment of the nairoviral OTUs, including HAZV (accession No. AAZ38668.1), CCHFV (accession No. AAZ98866.2), Nairobi sheep disease virus (NSDV; accession No. ACH99799.1), Dugbe virus (DUGV; accession No. AAB18834.1), Kupe virus (KUPEV; accession No. ABY82502.1), Erve virus (ERVEV; accession No. AFH89032.1) and Farallon virus (FARV; accession No. AMT75398.1). The residues forming the catalytic triad are outlined in black. Amino-acid positions targeted for mutation are indicated by red dots. The sequence alignment was generated using the *Clustal Omega* tool from EMBL–EBI (<https://www.ebi.ac.uk/Tools/msa/clustalo/>; Madeira *et al.*, 2019) and rendered with *ESPrpt* (<http://esprpt.ibcp.fr>; Robert & Gouet, 2014).

the altered binding orientation (Fig. 2). The first area is a 'selectivity pocket' with which Leu8 of Ub interfaces with the OTU, which forms a critical part of the binding (Deaton *et al.*, 2016; Dzimianski, Beldon *et al.*, 2019; Fig. 2, I). OTUs with hydrophobic selectivity pockets are predicted to possess robust DUB activity (Dzimianski, Beldon *et al.*, 2019). As expected based on the previously reported activity of HAZV OTU (Dzimianski, Beldon *et al.*, 2019; Dzimianski, Scholte *et al.*, 2019), the composition of the pocket in HAZV OTU is highly hydrophobic, possessing a valine, a leucine and a threonine at positions 18, 120 and 131, respectively. Interestingly, position 129 of HAZV OTU consists of a glycine, making it unique compared with other structurally characterized OTUs. The other nairoviral OTUs encode residues possessing a side chain at this position, such as the alanine in CCHFV OTU, that spatially constrain the location of Leu8 binding. In contrast, Gly129 in HAZV OTU could permit more freedom of motion, allowing Leu8 to potentially slide across the hydrophobic surface and adopt a tilted orientation. A second area where HAZV OTU appears to be more favourable for this mode of binding is in the $\alpha 3$ 'selectivity'

helix, which shows some of the greatest structural variation between OTUs (Capodagli *et al.*, 2013; Dzimianski, Beldon *et al.*, 2019; Fig. 2, II). In HAZV OTU this helix concludes with the residues Ala-Ala-Thr and is positioned in a way that leaves space for Ub to rest against it. In contrast, CCHFV OTU possesses Arg-Leu-Val at these positions. Combined with an inward shift of the helix in CCHFV OTU compared with HAZV OTU, this presents a more sterically restricted space that could prohibit Ub from adopting the same orientation as observed in the HAZV OTU complex. The third region consists of the $\beta 1$ - $\beta 1a$ turn in the OTU (Fig. 2, III). In HAZV OTU Ser13 and Asp14 point away from the interface, while in CCHFV OTU Ile13 occupies more space, presenting a steric boundary to the positioning of Ub.

3.2. Altering the enzyme activity and substrate specificity of HAZV OTU

Nairoviral OTUs possess a range of enzymatic activities and differ in the specific substrates that they most prefer. While CCHFV OTU, for example, possesses dual activity for both

Ub and ISG15, HAZV OTU is specific for Ub and ERVEV OTU is specific for ISG15 (Akutsu *et al.*, 2011; Capodagli *et al.*, 2011, 2013; Deaton *et al.*, 2016; Dzimianski, Beldon *et al.*, 2019; James *et al.*, 2011). The HAZV OTU-Ub structure led us to ask whether specific features are present that are responsible for its lack of deISGylase activity and whether mutations could be made to introduce this activity. Examination of the structure suggested five residues as potential targets: positions 13, 80, 120, 129 and 149 (Fig. 3a). In OTU-ISG15 structures, the OTU $\beta 1$ - $\beta 1a$ turn, and residue 13 in particular, is in proximity to a tryptophan in ISG15 (Fig. 3b). HAZV OTU contains a serine at this position that would have a largely neutral effect on such an interaction. In CCHFV OTU this residue is an isoleucine, which provides a hydrophobic surface that would be conducive to the tryptophan. ERVEV OTU possesses a glutamate at this position that could be potentially disruptive, but also presents the possibility of transient electrostatic interactions with residue 125, which is typically a serine or threonine in ISG15. The selectivity helix, which includes residue 80, has been shown to have a significant role in determining whether an OTU possesses ISG15 activity (Capodagli *et al.*, 2013; Dzimianski, Scholte *et al.*, 2019). In ERVEV and KUPEV OTUs specifically, residue 80

Figure 2

Comparison of the interface between Ub-bound HAZV and CCHFV OTU structures. Insets I-III show close-up views of the main regions of difference between the structures. Ub is shown in tan and bronze for the HAZV and CCHFV OTU structures, respectively. HAZV OTU is coloured cyan and CCHFV OTU is coloured burgundy.

(83 in ERVEV OTU) encodes an isoleucine that creates an important hydrophobic environment for a proline in ISG15, while HAZV OTU possesses an alanine that would interact to a lesser degree (Fig. 3c). Both residues 120 and 129 are part of the selectivity pocket, which has generally hydrophobic features in OTUs possessing Ub activity, while also possessing polar or charged residues in OTUs that interact with ISG15 (Fig. 3d). Residue 149 has been observed to facilitate interactions between OTUs and ISG15 (Dzimianski, Scholte *et al.*, 2019; Fig. 3e).

Making individual mutations at each of these sites produced notable, but limited, results (Fig. 4). Mutating Ser13 to both isoleucine and glutamate retained low ISG15 activity, but resulted in either a reduced or an improved Ub activity by 35% and 44%, respectively. Mutating Ala80 to isoleucine, on the other hand, was able to slightly improve the ISG15 activity while suffering a decrease in Ub activity similar to the S13I mutant. Interestingly, mutating Leu120 to threonine or valine produced widely divergent results. Incorporating a polar threonine completely abolished activity for both Ub and ISG15, while retaining hydrophobicity with a valine only reduced activity for ISG15. This may be indicative of a fundamental incompatibility of a polar amino acid in this position given the different orientation of substrate binding, or a disruption to the local structure by the reduced hydrophobicity. As expected, mutating Gly129 had a large impact on the activity, as this is most likely to require the substrate to adopt a different orientation upon binding. Mutating to alanine and serine reduced the activity towards Ub by 87% and

Figure 3
Comparison between the HAZV OTU-Ub complex and structures of the CCHFV and ERVEV OTUs bound to ISG15. (a) Side-by-side comparison of the complexes (CCHFV OTU-C-human ISG15, PDB entry 3phx; ERVEV OTU-C-mouse ISG15, PDB entry 5jze). Residues of specific interest in HAZV OTU are indicated with red dots. (b) Close-up views of the $\beta 1$ - $\beta 1a$ turn. (c) Interfaces formed with the selectivity helix of each structure. (d) Region forming the selectivity pocket. (e) Area surrounding residue 149 in each OTU complex.

95%, respectively. For ISG15, however, the G129S mutant showed a small boost in activity. Mutating Leu149 to polar or charged residues, including arginine, glutamine, asparagine and tyrosine, did not produce a substantial boost to ISG15 activity, while reducing the Ub activity significantly. Of the mutants, L149Y was the least disruptive to Ub activity, with just a 38% reduction.

Given the limited impact of individual mutations on the activity towards ISG15, we investigated whether incorporating multiple mutations could have an additive effect. Surprisingly, an A80I/G129S double mutant created a dramatic increase in ISG15 activity, which increased from almost negligible to a level ~60% of that reported for CCHFV OTU (Capodagli *et al.*, 2011, 2013; Dzimianski, Beldon *et al.*, 2019). Interestingly, incorporating L149Y to form a triple-mutant OTU more than doubled the activity compared with the double mutant. The non-additive, synergistic effect of these mutations suggests a repositioning of the substrate in the binding interface owing to G129S, which alters the relative importance of individual residue contacts. Consistent with this, attempting to improve the Ub activity by adding S13E, which individually increases Ub activity, to each of these mutants not only failed to do so, but also completely eliminated all activity.

Overall, these mutants demonstrate the feasibility of engineering the activity of OTU towards Ub and ISG15 through structure-guided mutagenesis. This includes, to our knowledge, the first report of substantial ISG15 activity being introduced into a viral OTU that previously lacked any such activity. These mutants also present themselves as potential tools to further investigate the mechanistic involvement of DUB/deISGylase activity in nairoviral infections. Currently, such studies have been limited to mutant OTUs in a CCHFV reverse-genetics systems (Bergeron *et al.*, 2015; Scholte *et al.*, 2017, 2019), which requires BSL-4 containment facilities. The recent development of an HAZV reverse-genetics system (Fuller *et al.*, 2019) presents a BSL-2 option to which these mutants could be applied. This presents opportunities to investigate the role of viral DUB/deISGylase activity in a manner that is more accessible to the field.

3.3. Inhibition of HAZV OTU by UbV-CC4

In addition to serving as an alternative to CCHFV for basic virology studies, HAZV has also been suggested as a disease model in immunodeficient mice (Dowall *et al.*, 2012). Ideally, from a disease-model standpoint, the antiviral compounds used within this system should closely mimic the effect that occurs in CCHFV. A recently developed inhibitor of CCHFV OTU activity is the Ub variant CC4 (UbV-CC4) identified through phage display (Zhang *et al.*, 2017). This inhibitor is able to inhibit CCHFV replication through its high affinity for the OTU, but is highly specific for CCHFV over other nairoviral OTUs (Scholte *et al.*, 2019). Specifically, of the 13 non-CCHFV nairoviral OTUs that were tested, only three were measurably inhibited. Although HAZV OTU shows the greatest degree of inhibition within this group, it has an IC_{50} that is ten times weaker compared with CCHFV OTU. With

the newly solved structure to hand, this led us to ask whether the HAZV OTU–Ub structure could provide insight into the underlying cause of these differences and the prospects for improving inhibition of HAZV OTU by rational drug design.

To evaluate the potential for improving inhibition, the structure of UbV-CC4 was superimposed on Ub in the HAZV OTU complex in order to assess the candidate residues for

Figure 4
Altering the enzymatic activity of HAZV OTU. (a) Structure of the HAZV OTU–Ub complex, with residues targeted for mutation indicated with red dots. (b, c) Activity of HAZV OTU mutants towards Ub-AMC and ISG15-AMC, respectively.

structure-guided mutagenesis (Fig. 5a). Examination of the predicted interface suggested three residues to target in

UbV-CC4: Thr9, Gln49 and Tyr68. Thr9 and Gln49 were mutated to valine or leucine residues to improve the potential for hydrophobic packing, and Tyr68 was mutated to histidine to resemble Ub (Fig. 5b). Interestingly, mutating Thr9 or Gln49 to leucine did not have an appreciable effect, while the Y68H mutation yielded less inhibition (Fig. 5c). This carried over to combination mutants, with a T9V/Q49L mutant only slightly increasing the inhibition, while the Y9L/Q49L and Q49L/Y68H mutants yielded less inhibition. This indicates that the main binding interface of UbV-CC4 is already highly optimized, suggesting that the difference in inhibition is likely to stem from interactions by the flexible tail. Unfortunately, the different substrate-binding orientations in HAZV OTU and CCHFV OTU prevent this region from being directly analysed based solely on the structures. The data suggest, however, that efforts to optimize the inhibition of CCHFV- and HAZV-like nairoviruses should focus predominantly on variants of this tail region. It can be envisioned that a library of UbV-CC4 tail variants could be constructed for screening to rapidly identify potential lead compounds for optimization.

4. Conclusion

The structure of HAZV OTU bound to Ub reveals insights into the diversity with which nairoviral OTUs engage their substrates. Utilizing structure-based mutagenesis, we were able to flip the substrate preference of the OTU from Ub to ISG15 and create mutants with eliminated or varying levels of activity for both substrates. Additionally, we explored the limitations of structure-guided mutagenesis in the improvement of Ub-derived OTU inhibitors. These insights present opportunities to utilize HAZV as a model to further elucidate the role of OTU DUB/deISGylase activity in nairoviral replication, including potential applications to CCHFV.

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Figure 5
Inhibition of HAZV OTU activity by UbV-CC4. (a) Overlay of UbV-CC4 onto the HAZV OTU-Ub complex based on a structure of UbV-CC4 bound to CCHFV OTU (PDB entry 5v5g). UbV-CC4 was overlaid on Ub based on the secondary structure of the core domain. I-III show the major regions of interest. (b) Sequence alignment of Ub and UbV-CC4. (c) Inhibition of HAZV OTU activity towards Ub-AMC by UbV-CC4 and UbV-CC4-derived proteins. Data are shown as percent activity relative to HAZV OTU without inhibitor.

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