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Structural basis of substrate recognition and covalent inhibition of Cdu1 from *Chlamydia trachomatis*

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Abstract

Based on the similarity between the active sites of the deubiquitylating and deneddylating enzyme ChlaDub1 (Cdu1) and the evolutionary related protease adenain a target-hopping approach screening on a focused set of adenain inhibitors has been pursued. The thereby identified cyano-pyrimidine based inhibitors represent the first active-site directed small molecule inhibitors for Cdu1. High-resolution crystal structures of Cdu1 in complex with two covalently bound cyano-pyrimidines as well as with its substrate ubiquitin have been obtained. These structural data were complemented by enzymatic assays and covalent docking studies to provide insight into Cdu1s substrate recognition, active site pocket flexibility and potential hotspots for ligand interaction. Combined, these data provide a strong foundation for future structure-guided medicinal chemistry optimization of this cyano-pyrimidine based scaffold towards more potent and specific Cdu1 inhibitors.

Author Contributions

Y.A.R. and C.K. designed the study; Y.A.R. (X-ray crystallography, enzymology, docking) and T.B.A. (docking, cheminformatics) conducted the research; E.A. provided the adenain inhibitors; T.K. and F.S. set up the enzymatic assay and provided Ub-PA; C.T. generated the Cdu1_CT construct. A.S. and S.G.K. provided the VME library compounds; E.A., C.S. and C.K. contributed to research design and data analysis; Y.A.R. and T.B.A. wrote the manuscript, with contributions from E.A., C.S. and C.K.

Supporting information for this article and the ORCID identification numbers for the authors can be found under: (Link to SD)

Covalent death sentence: Bacterial deubiquitylating enzymes (DUBs) can be utilized to enhance their pathogenicity. In our study, we have successfully harnessed the structural similarities of the active site of the chlamydial deubiquitylase 1 (Cdu1) with an evolutionarily related protease to discover the very first active-site covalent Cdu1 inhibitors. We demonstrate the molecular basis of Cdu1 ubiquitin recognition and establish criteria for the structure-informed medicinal chemistry optimization of potential Cdu1 specific inhibitors.

Keywords

CE proteases; covalent reversible inhibitor; DUB inhibition; target-hopping; cyano-pyrimidines

Introduction

Chlamydia trachomatis (CT) is an obligate intracellular gram-negative bacterium responsible for major genital and eye diseases in humans. With more than 130 million new cases per year, it is the most commonly diagnosed sexually transmitted infection in the world^[1]. Although CT infections can currently be treated with protein synthesis inhibitors such as azithromycin, doxycycline or tetracycline^[2], the bacterium is known to react to stress factors like antibiotics and starvation by differentiating into non infective aberrant forms, which are viable, metabolically active, resistant to antibacterial drugs and may reverse into the infective forms even months after the patient is in remission^[3].

Furthermore, there are indications that CT can readily develop resistance to front-line drugs when exposed to sub-inhibitory antimicrobial concentrations. A viable *Chlamydia suis* strain, which acquired tetracycline resistance by horizontal transfer, has already been isolated from diseased pigs^[4]. This emphasizes the ability of the members of the *Chlamydia* genus to acquire antibiotic resistance upon sufficient selective pressure.

CT has devised a variety of ways to evade the human immune response^[5]. As ubiquitylation and deubiquitylation regulate many key cellular processes such as protein degradation, cell cycle progression, transcriptional regulation, receptor internalization and signal transduction, it has been proposed that the chlamydial effector proteins ChlaDUB1 (Cdu1) and ChlaDUB2 (Cdu2) play a role in the infection mechanism and pathogenicity through the alteration of the ubiquitin-proteasome pathway^[6]. The Cdu1 cysteine protease permits the bacterium to

bypass the human host cell's inflammatory response regulated by NF- κ B through deubiquitylation of its inhibitory subunit, I κ B α ^[7]. Cdu1 also enables CT to interfere with the programmed cell death response of the infected host cells by impairing the proteasome-mediated degradation of the apoptosis inhibitor, Mcl1. A chlamydial transposon insertion mutant in the Cdu1 encoding gene leads to the loss of interaction between the chlamydial inclusion and Mcl1, stimulating the apoptotic response of infected human cells. Additionally, a significant reduction of replication is observed when this Cdu1-deficient CT strain infects fallopian tube cells (known as Fimb cells), the natural infection site of *C. trachomatis*^[8]. One may thus hypothesize that inhibiting Cdu1 could mitigate the proliferation of the pathogen while promoting the immune response of infected cells and thereby represents an attractive drug target.

Cdu1 belongs to a group of evolutionary related proteins known as the CE clan of cysteine proteases, which can be further divided into distinct families according to their differences in the catalytic domains^[9]. The CE clan features deubiquitinylase enzymes (DUBs) that can hydrolyze conjugates of ubiquitin (Ub) as well as Ubl-specific proteases (ULPs) which catalyze the cleavage of ubiquitin-like proteins (Ubl) such as Nedd8 and SUMO^[10]. In eukaryotes, the CE fold is present in peptidases responsible for maintaining free ubiquitin levels, rescuing proteins from ubiquitin-mediated degradation, and controlling the dynamics of ubiquitin-mediated signaling by cleaving UbIs from diverse substrates^[11]. In contrast, bacteria and viruses encode CE effector proteins to bypass the eukaryotic host cell defense mechanisms^[12]. These enzymes include not only deSUMOylases^[13] and deubiquitinylases^[14], but also acetyltransferases^[15]. The versatility of the CE fold can be attributed to the presence of a constant region near the conserved α -helix containing the active-site cysteine, which is surrounded by 3 variable regions (VR-1, 2 and 3). These variable regions are proposed to be responsible for granting substrate specificity. For instance, removal of Cdu1 VR-3, which is a unique α -helical feature of chlamydial DUBs, impairs binding to ubiquitin^[16].

Because of their key roles in various critical pathways, DUBs have recently garnered significant attention as drug targets. DUBs contain a well-defined active site mostly with a catalytic cysteine^[17], making them prone to inhibition by covalent modification of the active-site residue. Covalent inhibitors may have advantages over classic non-covalent inhibitors such as increased potency and longer residence times. Additionally, it may be difficult for the pathogen to develop resistance by modification of the active-site residue without compromising the enzymatic function^[18]. Even though the development of DUB inhibitors is still in its infancy, potent covalent and non-covalent active site inhibitors have been developed for some DUBs such as USP7^[19] and USP9X^[20]. Remarkably, USP9X shares Mcl1 as a substrate with Cdu1^[21].

To the best of our knowledge no inhibitors of Cdu1 have been described up to date. The adenoviral cysteine protease, adenain^[22], shares a high degree of similarity with Cdu1. The RMSD between the two proteins is 3.4 Å for the C α atoms of 158 residues (15% identity, DALI^[23]). Adenain plays a crucial role in the adenovirus lifecycle by facilitating the entry of viral genetic material into the human host cell and, thus, represents an attractive

therapeutic target^[24]. Interestingly, this protease has also been reported to possess DUB activity^[25].

Taking advantage of the high topological similarities between the active site of Cdu1 and adenain, a target-hopping approach has been pursued and a focused set of covalent adenain inhibitors, developed by Novartis^[26], has been screened utilizing a DUB enzymatic assay against Cdu1. X-ray cocrystal structures of the thereby discovered Cdu1 inhibitors confirmed that these compounds covalently label the active site Cys345 and provided insight into details of their binding mode. These cocrystal structures form the basis for further optimization of these compounds to more potent and specific Cdu1 inhibitors.

Results and Discussion

A structural alignment^[23] of Cdu1 and adenain revealed striking similarities between both proteins (Figure 1A). These similarities are not limited to the overall fold and spatial distribution of the secondary structure elements, but also include the topology of the active sites which are remarkably conserved (Figure 1B). To date, adenain is the only CE protease which has been the subject of a structure-informed drug design campaign, making its covalent inhibitors an attractive starting point for the development of ligands for other CE proteases. We initiated our study with the hypothesis that the homology between adenain and Cdu1 offers the possibility to identify primary features for the development of specific Cdu1 covalent inhibitors based on the already known adenain inhibitors.

Screening of potential Cdu1 covalent ligands

The first step towards the evaluation of Cdu1 covalent ligands was the identification of suitable and standardized enzymatic assay conditions. The second-generation DUB substrate Ub-Rh110Gly was used based on its higher dynamic range and unlikeliness to interfere with the fluorescence of test molecules, as compared to other ubiquitinated substrates^[27]. The dependency of Cdu1's enzymatic activity on the Ub-Rh110Gly concentration was explored by titrating 0.3 nM enzyme with increasing concentrations of substrate. This enzyme concentration displayed a linear phase of at least 2 min reaction time in control experiments. In contrast to the Cdu1 (155-401) construct used for crystallization, the Cdu1 (125-401) variant proved to be superior with respect to replicate tests. However, the longer construct did not display a different catalytic activity compared to the shorter construct. Most likely it was more stable in solution compared to the shorter construct. Michaelis–Menten kinetic analyses of the initial reaction velocities yielded a K_M of 1.7 μ M and a V_{max} of 179.4 RFU/s (Figure 2A). This K_M value is comparable to the CE clan deubiquitylase SseL (*Salmonella typhimurium*), with a K_M of 1.5 μ M for ubiquitin-1-amido-methyl-coumarin (Ub-AMC)^[14].

A set of 7 adenain inhibitors previously reported in the literature^[26] (Figure 1C) with different covalent warheads (one vinyl- sulfone, two cyano-pyrimidines and four glycine nitriles) were screened against Cdu1 (Figure S1-A, B). Additionally, a covalent tethering method was pursued^[28]. This method has already proven effective for identifying weak covalent binders for other proteases^[29] which might serve as starting point for inhibitor design. Here, a library of 180 thiol-reactive vinyl-methyl-ester (VMEs) compounds was analyzed (Table S4). Remarkably, only cyano-pyrimidines **3** and **5** yielded more than 50% of

inhibition at a concentration of 100 μM after 1.5 h incubation with Cdu1 (Figure S1-C, D) suggesting that the cyano-pyrimidine warhead might be a suitable electrophile to covalently label the active site Cys345.

IC₅₀ determination and reversibility assay

Reversible binding of cyano-pyrimidines **3** and **5** could be demonstrated by a rapid dilution assay, in which Cdu1 was pre-incubated with a 10-fold excess of the equivalent IC₅₀ for each compound to ensure loss of enzymatic activity. After rapid-dilution into assay buffer containing the substrate, DUB activity was restored within 2 hours. The lag phase during the first 30 min of the progression curve followed by a linear phase (Figure 2B) indicates a slow reversible binding, as Cdu1 recovers its activity while the inhibitors gradually dissociate^[30]. The compounds were found to have a modest potency against Cdu1, with IC₅₀ values of 204 μM and 113 μM for **3** and **5**, respectively (Figure 2C), in contrast to 0.06 μM and 24 μM for adenain.

The Cdu1~cyano-pyrimidine complexes and pose reproduction by covalent docking.

Despite their modest potency, crystal structures of **3** (6FDU) and **5** (6FDQ) in complex with Cdu1 could be obtained and solved to a resolution of 2.3 Å (Table S1), demonstrating that the reactivity of a cyano-pyrimidine warhead is sufficient to form a covalent bond to Cys345 in Cdu1 (Figure 3A). The electron density for **5** is better defined in chain A of our structure, while **3** is only visible in chain B. The stabilizing effect caused by a hydrogen bond of the thioimide group of the reaction product to Gln338 might play a crucial role. This hydrogen bond is clearly established in the crystal structure and the docking pose of **3** (Figure 3B, right). For **5**, the docking result predicted a weaker hydrogen bond with an unfavorable geometry, whereas the crystal structure exhibits optimal spatial positioning of the thioimide for this interaction (Figure 3B, left).

The spatial features of Cdu1's substrate binding pocket permit an almost planar arrangement of the thioimide group and the pyrimidine ring. Such a conformation represents an extended delocalized system upon reaction and contributes to the stabilization of the reaction product.

An intermolecular interaction especially important for stabilizing the binding to Cdu1 is a hydrogen bond donated by Trp247. **3** and **5** are able to establish this interaction through a carbonyl group of the amide close to the pyrimidine ring. The crystal structures as well as the docking poses of **3** and **5** feature this hydrogen bond. Another important interaction observed for **3** is a hydrogen bond donated by the main chain nitrogen of Ser168, which was also predicted by our docking studies.

The electron density of the benzene ring of **5** and the dichloro-benzene ring of **3** was not completely defined in the X-ray structures (Figure 3A). These might reflect flexibility of these parts of the ligands within the active site of Cdu1 (Figure S2-B). The aryl group in **5** might be temporarily stabilized by a weak van der Waals contact with Ala266. The same may be the case for the dichloro-benzene ring of **3**, which is mainly solvent exposed.

A detailed analysis of the interaction patterns and conformations in the crystal structures of **3** and **5** provide a basis for our design efforts towards the identification of more potent and specific cyano-pyrimidine based inhibitors. The structures permit the calibration of a Cdu1-optimized covalent docking procedure to quickly predict the binding orientation of cyano-pyrimidine ligands in the binding pocket of Cdu1. Via crystal pose reproduction studies on **3** and **5** using GOLD's^[31] covalent docking routines and by probing all scoring functions available in GOLD and different sampling settings, the empirical ChemScore scoring function^[32] was found to be the most reliable model to predict the key interactions and ligand conformations observed in the experiment. ChemScore correctly predicted the key hydrogen bond to Trp247 whereas the other GOLD scoring functions failed consistently.

To decide with more confidence on the final representative docking pose to be compared with the experiment, we relied not only on ChemScore, but also included the predictive power of the DSX scoring functions^[33] by rescoring the 50 best GOLD poses and calculating a consensus rank (cf. Experimental Section). The thus selected, representative docking poses deviate from the experiment by an RMSD of 1.04 Å (rank 2) and 0.93 Å (rank 1) for **3** and **5**, respectively (cf. SD Docking and scoring section for further details about the obtained poses).

Cdu1's pocket flexibility

The Cdu1~cyano-pyrimidine bound structures also reveal important features with respect to the flexibility of the substrate binding site. In contrast to **5**, which is *para*-substituted at the cyano-pyrimidine ring, **3** is *meta*-substituted. To establish the observed hydrogen bond with Trp247, the warhead on **3** is forced to move towards Val241. To avoid a steric clash, the flexible loop between Cdu1's α -helix D and β -strand 2 moves up towards an open position (Figure 4A, depicted in gray), thus breaking the internal hydrogen bond between Val271 and Ser274, which otherwise stabilizes a closed conformation (Figure 4A, depicted in orange). The presence of the bulky iso-pentyl substituent in **3** might also contribute to the loop opening, breaking an additional hydrogen bond between Ala266 and Gly269. The flexibility of α -helix D is also reflected in high temperature factors for this region as compared to the rest of the protein (Figure S2-B).

Cross-target specificity of cyano-pyrimidines **3** and **5**

As hypothesized, the cyano-pyrimidine warhead of both ligands binds to Cdu1 in a similar way as to adenain with an RMSD^[34] over the heavy atoms of the warhead of 0.56 Å for **3** and 1.57 Å for **5** (Figure 4B, right). There are, however, a few key differences in the binding modes beyond the covalent warhead. The terminal aromatic ring of **5** establishes a van der Waals contact with adenain's Val53, moving the ligand outside of the substrate binding site (Figure 4B, top-center). In contrast, this ring establishes the same type of interaction with Cdu1's Ala266, keeping the ligand below α -helix D and positioning the ligand's carbonyl group at an optimal distance to form a hydrogen bond with Trp247 (Figure 4B, top-left). The backbone of cyano-pyrimidine **3** addresses Cdu1's Glu338, Trp247 and Ser168 through hydrogen bonds (Figure 4B, bottom-left). This is analogous to how the ligand interacts with adenain's Glu115, Asn44 and Glu5 (Figure 4B, bottom-center). It is worth to highlight that, unlike **5**, **3** has improved specificity for adenain^[26b]. In adenain, the terminal dichloro-

benzene ring is positioned within a hydrophobic pocket while establishing a weak halogen bond with Ala46. These features are absent in Cdu1 where the dichloro-benzene group is oriented towards the solvent and lacks shape complementarity with the pocket. This might be one of the factors explaining the considerable difference in potency of **3** between the two proteases. Interestingly, the 2-(S)-dimethylamino isomer of **3** seems to preferentially bind to adenain while the 2-(R)-dimethylamino isomer binds to Cdu1, where the bigger α -helical insert above the active site impairs the dichloro-benzene ring to fit inside the pocket, as observed for the 2-(S) isomer in adenain (Figure 4B and S2-A). This different preference might be one way to increase inhibitor specificity.

The Cdu1~ubiquitin covalent complex

Binding of cyano-pyrimidines **3** and **5** occurs in the substrate binding channel of ubiquitin. To explore the interaction hotspots of substrate recognition, which might aid in our inhibitor design campaign the Dub-specific active site directed probe, Ubiquitin-Propargylamide (Ub-PA)^[35], was used to generate the Cdu1~ubiquitin covalent complex and solve the crystal structure to a resolution of 1.6 Å (Figure 5A). The flexible C-terminal tail of the Ub-PA reaches Cdu1's catalytic cysteine. The Cdu1~Ub interface is characterized by a buried surface area of approximately 1089 Å² (VMD, "calculate SASA" tool^[36]). Cdu1 residues Ile225 and Phe191 address the Ub Ile44 hydrophobic patch through van der Waals interactions with Ile44 and Val70. Remarkably, the side chain of Phe191 flips to make the binding pocket accessible for the C-terminus of ubiquitin. We previously hypothesized that Cdu1's α -helix D, which is unique for chlamydial DUBs, might play a role in substrate recognition by assuming a different orientation when the substrate is bound^[8]. However, our ubiquitin-bound structure shows that Cdu1 does not undergo significant conformational changes upon binding to ubiquitin apart from α -helix D tilting forward to address the Ub Ile36 patch through a hydrophobic contact with Ile267 (Figure 5B). This movement positions the backbone carbonyl of Cdu1 Gly269 in a favorable orientation to address the side chain of Ub Arg74 which is consistent with the reported inability of Cdu1 to bind ubiquitin upon deletion of α -helix D (i.e. VR-3 within the CE fold)^[16]. Moreover, the side chain of Ub Leu73 forms a van der Waals contact with Cdu1 Trp247, thereby disturbing the internal sulphur... π interaction^[37] between this residue and Met262, as observed in the apo-form (Figure 5B, inserted panel). Ub Ile73 can be described as a wedge that separates the Trp247-Met262 pair, which in turn facilitates the motion of the chlamydial α -helix D to permit proper binding to the substrate. Cdu1 Trp247, Ser168 and Asp167 play a role in recognizing the C-terminus of Ub by establishing hydrogen bonds with the backbone carbonyl of Arg74, Leu73 and Arg72, respectively. Additionally, the side chains of Cdu1 Asp169 and Glu395 assume conformations towards Ub Arg42 and Gln49 to further stabilize the complex.

Contacts with the hydrophobic Ub Ile44 and Ile36 patches are the most commonly observed DUB-Ub interaction motives. Both features are conserved in NEDD8, but not in other UbIs such as SMT3^[12]. Other conserved residues in NEDD8 include Arg42, Gln49, His68, Val70 and Arg74 (Figure 5C) – all of which are also present in ubiquitin and are important for the Cdu1-Ub interaction. Recognition of these conserved residues provides a molecular explanation of the reported deneddylating activity of Cdu1^[6]. Our observations thus suggest

that ubiquitin recognition is triggered by a concerted mechanism involving the interaction of both the C-terminus (Leu71 to Gly76) and the globular core of ubiquitin with key Cdu1 residues. This facilitates a conformational change on α -helix D to allow proper binding to the substrate. Since the key residues for the described interactions are conserved between ubiquitin and NEDD8 we assume that NEDD8 is bound to Cdu1 in a very similar way as observed for ubiquitin and Cdu1 may undergo similar movements to accommodate NEDD8.

Considerations for the design of specific Cdu1 covalent ligands

A comparison of the Cdu1~ubiquitin complex with the inhibitor bound structures reveals that the peptidic backbone of cyano-pyrimidine **3** reaches into the active site of Cdu1 in a similar way as the C-terminus of ubiquitin (Figure 6A). Both substrate and inhibitor address Cdu1 residues Gln338, Trp247 and Ser168, suggesting that these residues are hotspots for interactions. However, Gln338 is a conserved feature across the human SENP family while Trp247 is replaced either by an asparagine (SENP6, SENP7 and SENP8) or a histidine (SENP1, SENP2, SENP3, and SENP5) that can as well act as a hydrogen bond donor. The conserved nature of these residues might pose a challenge for the design of ligand specificity over other DUBs. This is especially the case for Ser168, where only the main chain nitrogen is required to establish an interaction. Therefore, addressing residues on the unique α -helix D of Cdu1 (i.e. VR-3) such as Met262, Ala266 and Gly269 may need to be preferentially taken into consideration for the design of new Cdu1 ligands. Interestingly, the side chain of Ub Arg74 and Leu73 are located at equivalent positions as the iso-pentyl substituent and the dimethylamine substituent in **3**, respectively. Modifying the substitution pattern of **3** to mimic Arg74 and Leu73 may improve the ligand towards complementarity and yield an increased potency. However, the loop opening triggered by **3** (described above) might represent a net energetic penalty which must first be compensated by the ligand upon binding. In contrast, the backbone of **5** (i.e., *para*-substituted with respect to the cyano-pyrimidine ring) keeps the active site closer to the Ub-bound conformation (Figure 6B), while addressing Trp247 and Ala266. This closed conformation may favor the backbone of **5** to initiate Cdu1 inhibitor optimization efforts.

Conclusions

Due to their relevance in the regulation of essential cell processes, the development of inhibitors for DUBs is an attractive, yet challenging research area^[17].

Exploiting the high active site homology between Cdu1 and the evolutionary related protease adenain by utilizing cross-target screening of protease inhibitors permitted the identification of the first two small molecules that bind to the active site of Cdu1. This target-hopping strategy resulted in successful repositioning of inhibitors and has delivered unprecedented starting points for Cdu1 inhibition. Our crystal structures of Cdu1 in complex with the inhibitors as well as the ubiquitin bound structure (PDB: 6FDK, 6FDU, 6FDQ) provide insights into the details of Cdu1 ligand recognition and binding pocket flexibility.

The interaction network of the C-terminus of ubiquitin and of the cyano-pyrimidines **3** and **5** indicate that cyano-pyrimidine ligands do not only covalently bind to Cys345 but have the potential to be optimized to potent covalent inhibitors for this bacterial deubiquitylase by

also addressing other hotspots for interactions in Cdu1's active site such as Asp167, Ser168, Trp247 and Gln338. Specificity for this bacterial DUB could be achieved by targeting residues located at the chlamydial α -helical insert of Cdu1 such as Met262, Ala266 and Gly269.

Our covalent docking model readily enables virtual screening of compound libraries as well as *in-silico* optimization of Cdu1 covalent binders, an effort which is currently being pursued but is beyond the scope of the work described herein.

Experimental Section

Cloning, Expression and Purification of Cdu1

The coding sequences for Cdu1 125-401 and 155-401 (numbering corresponding to Uniprot ID B0B9A0) were amplified from the pET28a/Cdu1 vector with Phusion polymerase (New England Biolabs) and cloned with the SLIC method^[38] behind the 3C-tagcleavage site of the pETM-14 vector (EMBL vector collection). The Cdu1_CT (155-401) variant in which three cysteines were mutated on the basis of sequence similarity across Cdu1s from different *Chlamydia* species (C174A, C226S and C386A) was generated by gene synthesis (GenScript). Cloning was pursued as described for the wild type variant.

Plasmids were transformed into *E. coli* BL21 (DE3) STAR cells. An overnight primary culture was inoculated into LB medium with the appropriate antibiotics and grown at 37°C to an OD₆₀₀ of 0.8. The culture was induced with 1.0 mM IPTG for overnight overexpression at 16°C.

After cell lysis, Cdu1 was purified by Immobilized Metal Affinity Chromatography (IMAC). The cell lysate was mixed with Ni-TED beads, incubated and washed with lysis buffer containing 25 mM HEPES (pH 7.5), 100 mM NaCl, 5 mM imidazole and 1 mM β -mercaptoethanol. The protein was subsequently eluted with the same buffer containing additionally 250 mM imidazole. Fractions containing Cdu1 were dialyzed overnight against 25 mM HEPES (pH 7.5), 100 mM NaCl and 1 mM DTT, while the His-tag was cleaved off by the addition of 3C protease. The protein was further purified by size exclusion chromatography (SEC) utilizing a Superdex 75 16/60 column (GE Healthcare) in a buffer containing 25 mM HEPES (pH 7.5), 100 mM NaCl and 1 mM DTT.

Three independent batches of the Cdu1 (125-401) variant used for enzymatic assays were purified as described above, each within a single day without over-night cleavage of the affinity tag to avoid loss of enzymatic activity due to oxidation of the active-site cysteine residue.

Generation of the Cdu1~ubiquitin covalent adduct

Ub-PA was prepared via a modified intein chemistry protocol^[39]. Ub(1-75) C-terminally fused to an Intein-CBD tag in the vector pTXB1, was expressed in *E. coli* BL21star (DE3) pRARE2 cells in TB-medium supplemented with 100 μ g/ml ampicillin and 34 μ g/ml chloramphenicol. After cell growth to an OD₆₀₀ of 2.5 at 37°C, expression was induced by the addition of IPTG to a final concentration of 0.3 mM and cells were harvested after

overnight incubation at 20°C. Cells were resuspended in lysis buffer (50 mM HEPES, 100 mM Na-acetate, pH 6.5) and lysed by sonication. The cleared lysate was applied to a 30 ml gravity flow chitin affinity column (New England Biolabs), incubated for 1 h, followed by a column wash with 2 × 100 ml lysis buffer. Intein mediated cleavage was performed by the addition of 30 ml lysis buffer containing 50 mM Na-β-mercaptoethanesulfonate (MESNa, Sigma Aldrich) over night at 20°C. The resulting eluate containing Ub(1-75)-MESNa (thioester) was then concentrated by ultrafiltration (Amicon, 3000 Da MWCO) and diluted ten-fold in lysis buffer supplemented with 250 mM propargylamine. After 3 h of incubation at room temperature, the reaction mixture was concentrated, and the resulting Ubi-PA was separated from excess PA/MESNa by SEC using a Superdex 75 16/60 column (GE) equilibrated with lysis buffer.

For the generation of the Cdu1-Ubi covalent adduct, the Cdu1_CT variant was incubated overnight at 4 °C with Ub-PA at a Cdu1:Ubi-PA molar ratio of 1:2. Complex formation was confirmed through SDS-PAGE and the fusion product was purified with a Superdex 75 10/300 column (GE Healthcare) using the same buffers as for wild type Cdu1.

Enzymatic assays

The enzymatic activity of Cdu1 was characterized using the fluorogenic substrate Ub-Rh110Gly^[27] (UbiQ Bio BV). The kinetic parameters were determined by monitoring the progression of substrate cleavage using a CLARIOstar plate reader (BMG LABTECH) with fluorescence excitation at 487 nm and emission detection at 535 nm in the presence of 20 mM NaCl, 20 mM HEPES (pH 7.5), 1 mM TCEP, and 50 µg/mL BSA at 28 °C. A substrate concentration response curve was plotted by titrating 0.3 nM Cdu1 (125-401) with increasing concentrations of Ub-Rh110Gly (0.25 to 5.0 µM).

The inhibition of Cdu1 activity by a set of 7 adenain inhibitors^[26] (Figure 1C) and 180 vinyl-methyl-ester (VME) fragment-like covalent binders for cysteine proteases^[28-29] (Table S4) was evaluated in 20 mM NaCl, 20 mM HEPES (pH 7.5), 1 mM TCEP, and 50 µg/mL BSA at 28°C. 0.6 nM Cdu1 (125-401) were incubated for 1.5 h at room temperature in the presence of 100 µM of each inhibitor (2% final DMSO concentration). The reaction was started by adding 20 µL of 500 nM Ub-Rh110Gly (final volume of 40 µL per well). This resulted in a final enzyme and substrate concentration of 0.3 nM and 250 nM, respectively. A well containing all components of the reaction except for the enzyme and the inhibitor was used as a blank. For the determination of the initial velocities, the data points corresponding to the first 2 minutes of the reaction were blank-corrected and plotted against time. The percentage of inhibition for each ligand was calculated as the ratio of the initial velocities for the inhibited reactions and a DMSO control (Figure S1).

Determination of the IC₅₀ values for cyano-pyrimidines **3** and **5** was performed by titrating 0.3 nM Cdu1 (125-401) with increasing inhibitor concentrations ranging from 3 to 3000 µM. After incubation for 1 h, the rate of Ub-Rh110Gly cleavage was measured in duplicate at a substrate concentration equivalent to the K_M for three independent protein batches. The dose response semi-logarithmic curves were fitted using GraphPad and the logistic function:

$$y = A1 + \frac{(A2 - A1)}{1 + 10^{(x - \log(IC_{50}))}}$$

where y is the % of inhibition at the inhibitor concentration x, A1 is the lowest inhibition value and A2 is the maximum inhibition value.

For the rapid dilution assay, the enzyme (100-fold concentrated compared to standard assays) was incubated with approximately 10-fold the IC₅₀-equivalent concentration of cyano-pyrimidines **3** and **5**. After incubation for 1 h at room temperature, samples were diluted 100-fold with assay buffer containing substrate, and the time course of substrate cleavage was monitored. In the enzyme control, Cdu1 (125-401) was mixed with DMSO before dilution into buffer-containing substrate. In the substrate control, no enzyme was used.

Protein Crystallization and X-Ray Data Collection

Crystallization of Cdu1 (155-401) was achieved overnight at 4°C through the hanging drop vapor diffusion method and using serial seeding as previously reported^[8]. Crystals were soaked in cryo protectant solution (100 mM Bicine pH 9.0, 10% PEG 20000, 2% 1,4-dioxane and 40% PEG 400) containing additionally 50 mM of cyanopyrimidine **3** (racemic), or 5 mM cyano-pyrimidine **5** for 65 h and 30 min, respectively. The Cdu1~Ub complex was crystallized at a protein concentration of 20 mg/ml utilizing the Cdu1_CT variant in 0.1 M MES (pH 6.5) and 12% PEG 20000.

All crystals were flash frozen in liquid nitrogen. Diffraction data were collected at the European Synchrotron Radiation Facility (ESRF), beam line ID23-1, Grenoble.

Structure Determination and Refinement

Data were integrated with XDS^[40] and scaled with AIMLESS^[41]. The structures of Cdu1~cyano-pyrimidine **3** and Cdu1~cyano-pyrimidine **5** were solved by molecular replacement in Phaser^[42] using the apo Cdu1 structure (PDB 5B5Q) as search model. The Cdu1~Ub complex was solved using the apo Cdu1 structure (PDB 5B5Q) and Ub (PDB 1UBQ) as search model. Manual model building was performed in COOT^[43] and model refinement was carried out with REFMAC^[44]. Data collection and refinement statistics are summarized in Table S1. All Figures representing crystal structures were prepared using PyMOL^[45].

Covalent docking

The crystal structures of the protein-ligand complexes of cyano-pyrimidines **3** and **5** (PDB: 6FDU, 6FDQ) were prepared for docking using MOE^[46]. After removal of ligands and water molecules MOE's protein structure preparation pipeline^[47] was applied to automatically assign tautomer and protonation states and optimize the hydrogen-bond networks. Protonate3D^[48] was internally invoked at pH 7.5. Cyano-pyrimidines **3** and **5** were additionally subjected to a MMFF94x^[49] geometry optimization in MOE (RMS gradient convergence criterion: 0.001 · kcal · mol⁻¹ · Å⁻¹). Post-reacted species with a

nucleophilic sulfur atom attached to the cyano group of cyano-pyrimidines **3** and **5** (required by GOLD) were manually prepared in MOE's Builder tool. The GOLD program^[31, 50] (v5.4.1) was used to dock the ligands under the constraint of a covalent link to Cys345. Non-default GOLD^[51] settings regarding enhanced ligand conformational flexibility, as well as the cavity definition can be found in SD, Docking and Scoring section. The empirical scoring function ChemScore^[32] was found to robustly model key intermolecular interactions and, hence, to yield the best crystal pose reproduction among all of GOLD's scoring functions. Pose reproduction performance was assessed by RMSDs between docked poses and crystal ligand structures calculated in GOLD. The 50 best ChemScore poses were rescored using the knowledge-based scoring function DSX^[33] (v0.89) (with CSD and PDB potentials (version 05/11); in the case of CSD potentials, torsion, intramolecular clash and SAS potentials were also activated). For selection of the best poses, a consensus ranking approach was devised by weighting the ranks of each of the 3 scoring functions equally: $1/3 \cdot (R_{\text{ChemScore}} + R_{\text{DSX_PDB}} + R_{\text{DSX_CSD}})$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Cartoon representations of Cdu1 (PDB: 5B5Q, green) and adenain (PDB: 1NLN red). In the overlay, the structures were aligned based on the cysteine-containing α -helix (catalytic triad depicted in gray, ball and stick representation). (B) 3D conservation mapping of the residues surrounding the active site cysteines of Cdu1 (blue) and adenain (magenta). Distances between equivalent atoms ($\text{\AA}$) are depicted in green. (C) Structures of adenain inhibitors 1 to 7^[26].

Figure 2.

(A) Michaelis-Menten analyses of Ub-Rh110Gly substrate cleavage in the presence of 0.3 nM Cdu1 (residues 125-401) (B) Rapid dilution assay. Time course of substrate cleavage: cyano-pyrimidine **3** ($n_{\text{rep}}=6$); cyano-pyrimidine **5** ($n_{\text{rep}}=6$); substrate control ($n_{\text{rep}}=3$). The DMSO control shows the uninhibited reaction ($n_{\text{rep}} = 6$). Error bars indicate the standard error of the mean. (C) Concentration-response curve for Cdu1 (125-401) activity after 1 hour of incubation with cyano-pyrimidine **3** (right) and cyano-pyrimidine **5** (left). The data represent the mean of at least 3 independent experiments performed in duplicate. * mixture of two diastereoisomers.

Figure 3.

(A) 2Fo – Fc (1.0 σ , blue) and Fo-Fc (3.0 σ , green) omit maps for cyano-pyrimidine **5** (Cdu1 chain A, left) and for cyano-pyrimidine **3** (Cdu1 chain B, right). (B) Comparison of the representative top-ranked docking poses (green) modeled with GOLD and a consensus-ranking approach with the experimentally observed binding modes (orange) of cyano-pyrimidines **3** (PDB: 6FDU, right) and **5** (PDB: 6FDQ, left). The docking poses of **3** (consensus rank 2) and **5** (consensus rank 1) differ by 1.04 and 0.93 Å from the corresponding experimentally observed poses.

Figure 4.

(A) Superposition of Cdu1 in complex with **3** (gray) and **5** (orange). The loop between α -helix D and β -strand 2 moves towards an open conformation to accommodate cyano-pyrimidine **3**. (B) Comparison of the experimental binding modes of cyano-pyrimidines **3** and **5** to Cdu1 (green, left column) and adenain (red, central column), and superposition of the complexes (right column). The R- and S-configurations of **3** are indicated in the lower left and the lower middle panel.

Figure 5.

(A) Crystal structure of the Cdu1~Ub complex (cartoon representation) and 2Fo-Fc map of the C-terminus of ubiquitin (Leu71-Arg74) within the active site of Cdu1 (1.0 σ , blue). **(B)** Superposition of the Cdu1 apo (PDB: 5B5Q, green) and the Cdu1-Ub complex structure (PDB: 6FDK, grey) illustrating the interaction network and the movement of α -helix D towards ubiquitin upon binding of the substrate. **(C)** Sequence alignment of ubiquitin, SMT3 and NEDD8. Key interaction spots between Cdu1 and ubiquitin (Ile36, Arg42, Ile44, Gln49, Val70 and Arg74) are also possible in NEDD8 at equivalent positions.

Figure 6. Superposition of the Cdu1~Ub complex with the Cdu1~cyano-pyrimidine **3** (A) and the Cdu1~cyano-pyrimidine **5** (B) complex, illustrating the flexibility of the active site upon ligand binding.