Structural and functional insights into the DNA damage-inducible protein 1 (Ddi1) from protozoa

Killivalavan Asaithambi 1, Iman Biswas 1, Kaza Suguna *

Molecular Biophysics Unit, Indian Institute of Science, Bangalore, 560012, India

ABSTRACT

Ddi1 is a multidomain protein that belongs to the ubiquitin receptor family of proteins. The Ddi1 proteins contain a highly conserved retroviral protease (RVP)-like domain along with other domains. The severity of opportunistic infections, caused by parasitic protozoa in AIDS patients, was found to decline when HIV protease inhibitors were used in antiretroviral therapy. Parasite growth was shown to be suppressed by a few of the inhibitors targeting Ddi1 present in these parasites. In this study, the binding of HIV protease inhibitors to the RVP domain of Ddi1 from *Toxoplasma gondii* and *Cryptosporidium hominis*; and the binding of ubiquitin to the ubiquitin-associated domain of Ddi1 from these two parasites were established using Biolayer Interferometry. The crystal structures of the RVP domains of Ddi1 from *T. gondii* and *C. hominis* were determined; they form homodimers similar to those observed in HIV protease and the reported structures of the same domain from *Saccharomyces cerevisiae*, *Leishmania major* and humans. The native form of the domain showed an open dimeric structure and a normal mode analysis revealed that it can take up a closed conformation resulting from relative movements of the subunits. Based on the crystal structure of the RVP domain of Ddi1 from *L. major*, a seven residue peptide inhibitor was designed and it was shown to bind to the RVP domain of Ddi1 from *L. major* by Biolayer Interferometry. This peptide was modified using computational methods and was shown to have a better affinity than the initial peptide.

1. Introduction

The DNA damage-inducible protein 1 (Ddi1) is a multidomain protein that was originally identified as the product of a gene which expressed both MAG1 (3-methyladenine DNA glycosylase) and Ddi1 under the control of a bidirectional promoter, in *S. cerevisiae* or yeast (Liu and Xiao, 1997). This protein belongs to a family of proteins known as ubiquitin receptors of the proteasome-mediated degradation pathway and take part in several cellular processes. Ddi1 degrades HO endonuclease and the F-box protein, Ufo1, to enable the regulation of cell cycle progression (Kaplun et al., 2006; Ivantsiv et al., 2006). By interacting with the t-SNARE and v-SNARE proteins, Ddi1 acts as a negative regulator of exocytosis (Lustgarten and Gerst, 1999; Marash and Gerst, 2003; Gabriely et al., 2008). In budding yeast, Ddi1 participates in S-phase checkpoint control by suppressing a temperature sensitive mutant of pds1 (Clarke et al., 2001). The transcription factors SKN-1A and Nrf1 (a homologue of SKN-1) were reported to be the natural substrates of Ddi1 in *Caenorhabditis elegans* (Lehrbach and Ruvkun, 2016) and human Ddi2 (a homologue of Ddi1) (Koizumi et al., 2016), respectively. Also, human Ddi1 and Ddi2 were found to maintain genome stability by removing RTF2 from replication forks (Rottemann et al., 2018). Ddi1 of yeast (yeastDdi1) was identified as a conserved metacaspase substrate (Bouvier et al., 2018). It has been found to assist in the removal of proteins from DNA-protein crosslinks, which hinder the replication process (Svoboda et al., 2019; Serbyn et al., 2020). YeastDdi1 was shown to cleave polyubiquitinated substrates, probably playing a role in compensating for the loss of proteasome function (Yip et al., 2020).

Ddi1 comprises three major domains: a ubiquitin-like (UBL) domain, a ubiquitin-associated (UBA) domain and a retroviral protease (RVP)-like domain; an additional domain preceding the RVP domain called the helical domain of Ddi1 (HDD), was identified in yeastDdi1 (Treppe et al., 2016) and humanDdi2 (Sivá et al., 2016). The domain organization of...
the proteins reported in the present study are shown in Fig. 1. Interestingly, the RVP domain is always present in Ddi1 but the UBA and/or UBL domain is absent in a few organisms (Nowicka et al., 2015; Krylov and Koonin, 2001; Sivä/C19a et al., 2016). The ubiquitin receptor family of proteins bind to ubiquitinated substrates through the UBA domain and deliver the substrates to the proteasome by interacting with the regulatory subunit of the 26S proteasome through the UBL domain. This facilitates the degradation of ubiquitinated proteins. The presence of the RVP domain makes Ddi1 unusual, as it is the only known protein among the ubiquitin receptor family of proteins to have this domain (Gabriely et al., 2008). A sequence comparison of Ddi1 from various organisms shows that the RVP domain is highly conserved. The NMR structures of the HDD domain revealed similarities to DNA binding domains from transcriptional regulators (Trempe et al., 2016; Sivä et al., 2016). The ubiquitin receptor family of proteins have this domain (Gabriely et al., 2008). A sequence comparison of Ddi1 from various organisms shows that the RVP domain is highly conserved. The NMR structures of the HDD domain revealed similarities to DNA binding domains from transcriptional regulators (Trempe et al., 2016; Sivä et al., 2016). Disorder prediction of Ddi1 revealed the presence of highly disordered regions between UBL and the HDD and between RVP and the UBA domains. This leads to high flexibility of the protein which may be important for the protein to mediate its functions associated with different domains (Aufderheide et al., 2015).

Interestingly, some of the AIDS patients treated by Highly Active Antiretroviral Therapy (HAART) acquired partial protection against leishmaniasis and other parasitic diseases. Later, this phenomenon was shown to be a consequence of the HIV protease inhibitors used in the therapy interacting with the Ddi1 present in parasites (Savoa et al., 2005; Valdivieso et al., 2007; Trudel et al., 2008; Li et al., 1998). It was reported that ritonavir and nelfinavir inhibit the growth of T. gondii which causes toxoplastic encephalitis (Wang et al., 2019). The protease activity of Ddi1 of T. gondii was found to be important for virulence in mice (Zhang et al., 2020). This has provided a new direction to develop specific inhibitors to Ddi1 to kill the pathogen. In case of severe immunosuppression following HIV infection, the use of antiretroviral therapy caused restoration of mucosal immunity due to an increase in CD4 T-cells leading to cure of clinical cryptosporidiosis (Schmidt et al., 2001). Nelfinavir could be an effective drug to treat multiple myeloma and other cancers due to its ability to inhibit the activity of human Ddi2 (Gu et al., 2020; Fassmannova et al., 2020).

The first structure of the RVP domain of Ddi1 to be reported was from yeast (yeastDdi1-RVP) (PDB Code: 2I1A; Sirkis et al., 2006). It was a homodimer, with each protomer having the retroviral protease fold; however, the loop region called the ‘flap’, which overhangs the active site could not be traced. In the second structure of the yeastDdi1-RVP (PDB Code: 4Z2Z; Trempe et al., 2016), the flap from one subunit of the dimer could be clearly defined as it interacts extensively with the N-terminal segment from an adjacent symmetry-related molecule, which mimics a substrate peptide and binds in the active site. In the structure of the RVP domain of human Ddi2 (humanDdi2-RVP) (PDB Code: 4RGH; Sivä et al., 2016), electron density could be seen for one of the flaps, which interacts with a symmetry-related molecule. In the structure of Ddi1-RVP from L. major (LeishDdi1-RVP) (PDB Code: SYQ8; Kumar and Suguna, 2018), the electron density corresponding to both the flaps was present; each flap of the dimer interacts with the active site residues of another dimer in the asymmetric unit via hydrogen bonds and salt bridges. Even though

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**Fig. 1.** Domain organization of Ddi1 from A) *C. hominis* and B) *T. gondii*. UBL, ubiquitin-like domain; HDD, helical domain of Ddi1; RVP, retroviral protease-like domain; UBA, ubiquitin associated domain.

**Fig. 2.** Interaction of the flap region with the active site of the neighboring dimer in the crystal structure of LeishDdi1-RVP.
Table 1

| Data collection and refinement statistics. Values in parentheses are for the highest resolution shell. |
|---------------------------------------------------------------|
| **Toxodi1-RVP (PDB id: 7Db6)** | **Cryptodi1-RVP (PDB id: 7EFY)** |
| Wavelength (Å) | 0.99 | 0.99 |
| Resolution range (Å) | 65.27–2.13 (2.18–2.13) | 47.44–2.80 (2.87–2.80) |
| Space group | P4₁2₁2₁ | P4₁2₁2₁ |
| Unit cell (Å) | a = b = 184.60, c = 184.34 | a = b = 81.55, c = 184.12 |
| Total reflections | 471379 (32340) | 35030 (5035) |
| Unique reflections | 54712 (4404) | 6686 (668) |
| Multiplicity | 8.6 (7.3) | 7.4 (6.5) |
| Completeness (%) | 100 (100) | 100 (100) |
| g < 3.0 (%) | 13.9 (3.1) | 11.1 (2.1) |
| Wilson R factor (Å²) | 42.16 | 81.30 |
| Rmerge (%) | 9.5 (67.8) | 9.5 (10.0) |
| Rfree (%) | 10.4 (15.2) | 10.9 (12.2) |
| RPDLS (%) | 4.2 (67.8) | 5.3 (6.0) |
| CC₁/₂ | 0.99 (0.37) | 0.99 (0.84) |
| Reflections used in refinement | 176969 (8844) | 4683 (222) |
| Rmerge/Rfree | 0.19/0.25 | 0.18/0.23 |
| RMSD bond (Å) | 0.0154 | 0.0064 |
| RMSD angles (°) | 1.833 | 1.497 |
| No. of atoms | Protein | 11178 | 852 |
| Glyceral | 96 | – |
| Water | 585 | 5 |
| Ramachandran map | Favored (%) | 95.93 | 100 |
| Allowed (%) | 4 | – |
| Outlier (%) | 0.07 | – |
| Average B factor (Å²) | Protein | 34.26 | 90.50 |
| Glyceral | 47.25 | – |
| Water | 41.35 | 73.30 |

A few of the Ddi1-RVP flap regions could not be traced in these reported structures, some of them could be clearly defined when this region interacted with other molecules in the crystal.

Though HIV protease inhibitors have detrimental effects on parasitic protozoa, they are not potent enough to be used for treatingeland diseases unless their efficacy is substantially improved. More structural insights on protozoal Ddi1 in the apo and inhibitor-bound forms will aid in this process. With this in view, we have taken up structure analysis of the RVP domain of Ddi1 from two protozoa, T. gondii and C. hominis. In addition, we performed a normal mode analysis (NMA) on both the structures and molecular dynamics (MD) simulation studies on the structure of the Ddi1-RVP domain from T. gondii to investigate the dynamics of this domain with a focus on the flap region. We also carried out in vitro binding studies of Ddi1 with HIV protease inhibitors using biolayer interferometry (BLI) and molecular docking of HIV protease inhibitors to the structures of Ddi1-RVP from T. gondii and C. hominis to probe Ddi1-inhibitor interactions. In this paper, we report the crystal structures of Ddi1-RVP from T. gondii and C. hominis; and binding of HIV-1 PR inhibitors, nelfinavir and saquinavir to Ddi1-RVP from T. gondii and C. hominis, respectively. This will provide a starting point for the development of antiprotozoal drugs with Ddi1 as the target.

In the structure of the RVP domain of Ddi1 from L. major (Kumar and Suguna, 2018), it was observed that four polypeptide chains are arranged as two dimers in the asymmetric unit (Fig. 2) and the flap of one of the subunits in one dimer interacts with the active site of another dimer in the asymmetric unit via hydrogen bonds and salt bridges. Based on this observation, with an anticipation that a peptide comprising seven residues (GYSYRQD) of the flap region interacting with the other dimer may act as an inhibitor to the protein, we studied the binding of this heptapeptide to the RVP domain of Ddi1 from L. major and modified this peptide which showed better binding.

2. Materials and methods

2.1. Cloning, protein expression and purification

The Ddi1 genes from T. gondii (Gene ID: 7893859, 1581 bp) and C. hominis (Gene ID: 3413713, 1152 bp) were synthesized by GenScript, USA. From the full length Toxodi1 gene, two constructs, Toxodi1-RVP + UBA (880–1551 bp) and Toxodi1-RVP (880–1263 bp) were subcloned in pET-28a vector between Nhel and Xhol sites with an N-terminal hexahistidine tag. From the full length Cryptodi1 gene, two constructs, Cryptdi1-RVP + UBA (595–1137 bp) and Cryptdi1-RVP (595–978 bp) were subcloned in pET-21b vector between Nhel and HindIII sites with a C-terminal hexa-histidine tag. The full length Ddi1 construct, LeishDdi1-f1 (1–390 aa) was cloned in pET-28a vector between Ndel and HindIII sites with an N-terminal hexa-histidine tag (Kumar and Suguna, 2018). The plasmids containing the gene of interest were transformed into E. coli BL-21 (DE23) competent cells and plated on LB agar medium containing kanamycin. A single colony of the transformed E. coli BL-21 cells was inoculated in LB medium containing kanamycin and grown at 37 °C for 10–12 h. One percent of the overnight culture was inoculated in LB medium and grown at 37 °C until the optical density at 600 nm reached ~0.6. Protein expression was induced by adding 0.25 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) and the cells were further grown at 18 °C for 16–20 h. The cells were harvested by centrifugation at 6000 rpm for 10 min. The pelleted cells were suspended in a lysis buffer containing 20 mM Tris/HCl (pH 7.5) and 300 mM NaCl. The cells lysed by sonication were centrifuged at 13000 rpm for 45 min. The supernatant was passed through a pre-equilibrated Ni-NTA column (GE Healthcare, Uppsala, Sweden), 20 column volumes of buffer containing 20 mM Tris/HCl (pH 7.5), 300 mM NaCl and 10 mM imidazole were passed through the column to remove impurities bound non-specifically to the column. The proteins were eluted with a buffer containing 20 mM Tris/HCl (pH 7.5), 300 mM NaCl and 300 mM imidazole. Further purification was carried out by gel filtration chromatography using a 16/60 Superdex 200 gel filtration column (GE Healthcare) equilibrated with a buffer containing 20 mM Tris/HCl (pH 7.5), 100 mM NaCl and 2 mM β-mercaptoethanol using a BioLogic DuoFlow FPLC system from Bio-Rad (Hercules, CA, USA). The purity of the proteins was examined by running 15% SDS-PAGE (Supplementary Fig. 1).

2.2. Activity assay and inhibition studies by fluorescence resonance energy transfer (FRET)

The activity assay was carried out using HIV protease substrate-1 (Sigma-aldrich). This is a synthetic peptide having the sequence Arg-Glu(EDANS)-Ser-Glu-Asn-Tyr-Pro-Ile-Val-Gln-Lys(DABCYL)-Arg. It contains the HIV protease cleavage site (Tyr-Pro) and two covalently modified amino acids for the detection of the proteolytic activity: the fluorophore EDANS was attached to the glutamic acid residue on the N-terminal side and the acceptor chromophore DABCYL to the lysine residue on the C-terminal side of the scissile bond. EDANS excited with light at 340 nm emits light at 490 nm which gets absorbed by the DABCYL fluorophore. The activity of the enzyme was determined by fluorescence resonance energy transfer (FRET).
Fig. 3. Computational workflow for the generation of a modified peptide.

- Protein + Initial Peptide complex
  - Select a random residue in the peptide and mutate it to other 19 amino acids and pick the residue which gives lowest ΔΔG<sub>binding</sub> for the complex.

- Protein + Peptide with mutation 1 complex
  - Select a random residue in the peptide and mutate it to other 19 amino acids and pick the residue which gives lowest ΔΔG<sub>binding</sub> for the complex.

- Protein + Peptide with mutation 2 complex

- Protein + Peptide with mutation n complex
  - n ≥ 10 times the no. of residues in the peptide

- Simulate the whole process for m times
  - m = 50

- The peptide which is the result for maximum times among the m no. of times is the resultant

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Fig. 4. Fluorescence intensity profiles of (A) ToxoDdi1-RVP and (B) CrysDdi1-RVP at pH 4.7 in the presence of HIV protease substrate-1.
2.3. Bio-layer interferometry (BLI)

The binding of HIV protease inhibitors to ToxoDdi1-RVP and CrypDdi1-RVP, binding of monoubiquitin and K48-linked diubiquitin to ToxoDdi1-RVP + UBA and CrypDdi1-RVP + UBA, and binding of peptides to LeishDdi1-fl were measured by BLI using an Octet Red96 system (Pall ForteBio, Fremont, CA, USA). To study the binding of HIV protease inhibitors, 10 μM of His-tagged protein was immobilized on the Ni-NTA
sensor tip which was then dipped in a solution containing 100 mM MES buffer (pH 5.5), 150 mM NaCl, 0.5% BSA, 0.05% Tween 20 and inhibitors at 75 μM, 150 μM, 300 μM and 600 μM concentrations for 3 min at each concentration. To study the binding of monoubiquitin and K48-linked diubiquitin, 1 μM of monoubiquitin/K48-linked diubiquitin was immobilized on the AR2G sensor tip via amine coupling mechanism and dipped in protein of varying concentrations in 20 mM HEPES (pH 7.5) and 100 mM NaCl for 10 min at each concentration. To study the binding of peptides to LeishDdi1-fl, 5 mM of peptide was immobilized on the AR2G sensor tip and dipped in protein of varying concentrations in 20 mM HEPES (pH 7.5) and 100 mM NaCl for 10 min at each concentration. Double referencing was performed in all the cases, to obtain more accurate results. A simple 1:1 Langmuir interaction model was used to fit the data.

2.4. **High performance liquid chromatography (HPLC)**

The enzymatic activities of ToxoDdi1-RVP and CrypDdi1-RVP were also probed by HPLC (Agilent Technologies, Compact LC, 1200) equipped with single wavelength UV-detector at 490 nm and an Eclipse C18 analytical column ((150 mm × 4.6 mm), particle size 3.5 μm)). Absorbance at a wavelength of 490 nm was recorded to detect the DABCYL group. The C-terminal fragment of the product should appear as a peak after cleavage. A reaction mixture consisting of 100 mM sodium acetate at pH 4.7, 1 M NaCl, 1 mM DTT and 22 μg/ml of ToxoDdi1-RVP/CrypDdi1-RVP and 10 μM of HIV protease substrate-1 was incubated at 37 °C for 1 h and was analyzed by HPLC. For the detection of the chromophore, 0.1% of TFA (Trifluoro acetic acid) and acetonitrile at 50:50 ratio were used as a mobile phase with the flow rate of 0.8 ml/min. Absorbance at 490 nm was also recorded for 2 μM of the substrate alone, as a reference.

2.5. **Kinetics assay**

Enzyme kinetics assay was performed using a reaction mixture consisting of 100 mM sodium acetate buffer at pH 4.7, 1 M NaCl and 1 mM DTT and 22 μg/ml of ToxoDdi1-RVP/CrypDdi1-RVP at increasing concentrations (2 μM, 4 μM, 6 μM, 8 μM, 10 μM, 20 μM, 40 μM, 60 μM, 80 μM and 100 μM) of HIV protease substrate-1. Cleavage of the HIV protease substrate-1 (Arg-Glu[EDANS]-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-Lys [DABCYL]-Arg) that occurs at Try-Pro was monitored. The reaction mixture was kept in a micro plate reader (Varioskan Flash; ThermoFisher Scientific) at 37 °C and the reading was taken every 60 s with 3 s stirring intervals for 60 min. The λ_{excitation} was 340 nm, and the λ_{emission} was 490 nm. A 96-well NUNC V-bottom polypropylene 0.45 ml plate (black) was used for this experiment. The constants K_m and V_max were calculated by fitting the data to the Michaelis-Menten equation by nonlinear regression.

2.6. **Site-directed mutagenesis**

To generate single mutants of ToxoDdi1-RVP and CrypDdi1-RVP, site-directed mutagenesis was carried out with the QuickChange Site-Directed Mutagenesis kit (Stratagene-Agilent technologies, Inc., Santa Clare, CA, USA). Using this kit, a single amino acid residue, the catalytic residue Asp315 in ToxoDdi1-RVP and Asp220 in CrypDdi1-RVP, was mutated to Ala (D315A/D220A) and Asn (D315N/D220N), and the mutations were confirmed by DNA sequencing. These four mutant proteins were expressed and purified in the same way as described for the wild-type protein. The purity of the mutants was checked by running 15% SDS-PAGE (Supplementary Fig. 1).

2.7. **Crystallization**

The microbatch method was employed for crystallization trials using the Hampton Research and Molecular Dimensions kits. 2 μl of the protein solution at 8–12 mg/ml concentration was mixed with 2 μl of various precipitants. The mixture was placed in the wells of the microbatch plate layered with paraffin and silicon oils (1:1) at 291 K. Crystals of ToxoDdi1-RVP construct were obtained at a protein concentration of 8 mg/ml in a condition containing 1.8 M ammonium citrate tribasic (pH 7.0) and 30% 2-propanol. Crystals of CrypDdi1-RVP construct were obtained at a protein concentration of 8 mg/ml in a condition containing 0.1 M MES-sodium hydroxide (pH 6.0), 30% polyacrylate sodium salt and 10% ethanol. Octahedron-shaped crystals of ToxoDdi1-RVP of length 0.1 mm and rectangular crystals of CrypDdi1-RVP of length 0.07 mm were obtained after 3–4 days.

2.8. **Data collection**

The X-ray diffraction data were collected from the crystals at XRD2 beamline using a Pilatus-6M detector at Elettra, Trieste, Italy, at a wavelength of 0.9918 Å. The crystals were cryoprotected with glycerol. The crystals of ToxoDdi1-RVP diffracted to a resolution of 2.1 Å; 480 frames were collected with an oscillation angle of 0.5° per image and a crystal to detector distance of 250 mm. The crystals of CrypDdi1-RVP diffracted to a resolution of 2.8 Å; 360 frames were collected with an oscillation angle of 0.5° per image and a crystal to detector distance of 270 mm.
2.9. Model building and refinement

The data were processed and scaled using *iMOSFLM* and *AIMLESS* of the CCP4 suite, respectively (Winn et al., 2011). The data collection and processing statistics are given in Table 1. The structures were determined by molecular replacement using *PHASER* (McCoy et al., 2007). The structures were manually built using *COOT* (Emsley and Cowtan, 2004) and refined by *REFMAC5* (Murshudov et al., 1997). The glycerol and water molecules were identified in the 2Fo-Fc and Fo-Fc electron density maps contoured at 1.0 and 3.0 σ levels, respectively. Several cycles of alternating model building and refinement were carried out until the $R_{work}$ and $R_{free}$ converged. Validation of the final structures was carried out using *MolProbity* (Chen et al., 2010) and the images were generated using *UCSF CHIMERA* (Pettersen et al., 2004). The atomic coordinates and structure factors were deposited in the Protein Data Bank with accession codes 7D66 for *ToxoDdi1-RVP* and 7EFY for *CrypDdi1-RVP*, respectively.

2.10. Molecular dynamics simulation and normal mode analysis (NMA)

An all-atom MD simulation of *ToxoDdi1-RVP* dimer was carried out using *GROMACS 5.0.4* with amber 99SB force field (Hornak et al., 2006), to investigate the dynamics of the flap region. The protein was solvated using the TIP3P water model (Jorgensen et al., 1983) and kept in a box generated such that the minimum distance between the protein and the edge of the box was 1.0 nm. To neutralize the net charge, 0.1 M NaCl was added to the system. A distance cutoff of 10 Å was used for van der Waals interactions. Energy minimization was carried out using the steepest descent method. The system was equilibrated at NVT ensemble followed by NPT ensemble for 100 ps. Temperature equilibration was performed using a modified Berendsen thermostat (Berendsen et al., 1984) with a coupling time constant of 0.1 ps and a reference temperature of 300 K. Pressure equilibration was performed using the Parrinello-Rahman method (Parrinello and Rahman, 1981) with a coupling time constant of 2 ps and reference pressure of 1 bar. A time step of 2 fs was used in the leapfrog integrator. The simulation was carried out for a period of 300 ns and the coordinates and energies were recorded every 10 ps.

NMA analysis was carried out using the elNemo server (Suhre and Sanejouand, 2004).

2.11. Design of a modified peptide inhibitor

The POS-SCAN module of the FoldX suite (Schymkowitz et al., 2005) was used to mutate every residue of the initial peptide (GVGRQEI) to all the nineteen other amino acid residues; the $\Delta \Delta G_{binding}$ value of the protein-peptide complex for each mutation was calculated. At a

![Fig. 7. BLI binding profile of inhibitors with (A) ToxoDdi1-RVP and (B) CrypDdi1-RVP.](image-url)
which the mutated to all the nineteen other amino acid residues; the mutation for step, a different residue of the peptide was selected randomly, and the protein-peptide complex has the lowest value was considered. In the next step, the AGGRESCAN (Conchillo-Sol et al., 2007) server suggested that the protein-peptide complex with the lowest energy protein-peptide complex. This constituted one simulation of the process. Multiple simulations were performed to avoid reaching a local minimum of the protein-peptide complex. The peptide which was the outcome of most of the simulations was considered to be the resultant peptide. The value of propensity for aggregation of the resultant peptide calculated using the AGRERSCAN (Conchillo-Solé et al., 2007) server suggested that the peptide was not prone to aggregation. The solubility of the peptide was checked using the INNOVAGEN-PepCalc tool.

2.12. Molecular docking

Molecular docking of ToxoDdi1-RVP with nelfinavir, CrypDdi1-RVP with saquinavir and LeishDdi1-RVP (PDB code: 5YQ8, Kumar and Suguna, 2018) with peptides was carried out using AutoDock 4.2 (Morris et al., 2009). The coordinates of nelfinavir and saquinavir were extracted from the crystal structures of their complexes with HIV protease (PDB Codes: 2R5Q and 4QGI, respectively).

2.13. Sequence alignment

Multiple sequence alignments were carried out using CLUSTAL Omega (Sievers and Higgins, 2018). A structure-based sequence alignment was carried out using the ESPript3 webserver (Gouet et al., 1999).

3. Results

3.1. Proteolytic activity of Ddi1 and its inhibition by HIV protease inhibitors

Though the inhibitory effect of HIV protease inhibitors on the growth of parasitic protozoa has been studied, no direct evidence of the proteolytic activity of Ddi1 or the inhibitory effect of these inhibitors directly on Ddi1 through in vitro studies has been reported until now. We have carried out a FRET-based activity assay using the synthetic substrate, HIV Protease Substrate-1 (Sigma-aldrich) of the ToxoDdi1-RVP and CrypDdi1-RVP constructs. An increase in the fluorescence intensity was observed with increasing protein concentration which implies an increase in the proteolytic cleavage of the substrate with increase in protein concentration. The fluorescence intensity did not increase for the reaction mixture containing 500 μM of the HIV protease inhibitor, nelfinavir with 20 μM of ToxoDdi1-RVP (Fig. 4A) and for the reaction mixture containing 500 μM of the HIV protease inhibitor, saquinavir with 20 μM of CrypDdi1-RVP (Fig. 4B). These results indicate that nelfinavir has an inhibitory effect on the proteolytic activity of ToxoDdi1-RVP and saquinavir has an inhibitory effect on the proteolytic activity of CrypDdi1-RVP as a result of which the substrate could not be cleaved.

The HPLC experiments illustrated the cleavage of HIV protease substrate-1 by both ToxoDdi1-RVP and CrypDdi1-RVP (Fig. 5). The substrate was completely cleaved and the labelled C-terminal peptide of the product appeared in the HPLC profile in the presence of these domains after 1 h of incubation. For CrypDdi1-RVP, both the substrate and the product were detected after an incubation period of 30 min, indicating that the reaction was in progress. The kinetics analysis (Fig. 6) showed a $V_{max}$ of 158.30 ± 18.49 FU/min and a $K_m$ of 16.72 ± 5.95 μM for ToxoDdi1-RVP and a $V_{max}$ of 159.10 ± 14.85 FU/min and a $K_m$ of 15.94 ± 4.58 μM for CrypDdi1-RVP. Compared to these domains, the full length LeishDdi1 displayed a better affinity with $V_{max}$ and $K_m$ values of 53.76 ± 1.60 FU/min and 0.288 ± 0.057 μM, respectively (Perteguer et al., 2013).

Single mutants, D315A and D315N of ToxoDdi1-RVP and D220A and D220N of CrypDdi1-RVP were generated (Supplementary Fig. 1) by mutating the catalytic residue Asp to Asn and Ala and activity assays were performed. As expected, no activity was observed (Fig. 6); this confirms the participation of Asp in the catalytic activity of these two domains as in other aspartic proteinases.

3.2. Binding of HIV protease inhibitors to Ddi1

In order to quantify the interaction of HIV protease inhibitors to Ddi1, we carried out in vitro binding studies of HIV protease inhibitors, saquinavir, nelfinavir, amprenavir, darunavir, indinavir and ritonavir to ToxoDdi1-RVP and CrypDdi1-RVP using BLI. Of all the inhibitors tested, nelfinavir showed binding to ToxoDdi1-RVP with a $K_d$ of 237.6 ± 6.3 μM and saquinavir showed binding to CrypDdi1-RVP with a $K_d$ of 242.1 ± 3 μM (Fig. 7 A,B, Supplementary Fig. 2). These affinities are slightly better than that for saquinavir binding to LeishDdi1-fl with a $K_d$ value of 314 ± 13 μM (Kumar and Suguna, 2018).
3.3. Binding of monoubiquitin and K48-linked diubiquitin to Ddi1

The binding of monoubiquitin and K48-linked diubiquitin to the UBA domain of yeast Ddi1 was reported by Trempe et al. (2016). In order to check the binding of monoubiquitin and K48-linked diubiquitin to the UBA domain of Ddi1 from *T. gondii* and *C. hominis*, we carried out in vitro binding studies using BLI. Monoubiquitin showed binding to the Toxo Ddi1-RVP + UBA construct with a $K_d$ of 38.5 ± 1.1 μM (Fig. 8A) and Cryp Ddi1-RVP + UBA with a $K_d$ of 37.6 ± 5.9 μM (Fig. 8B). These values are comparable to the $K_d$ value of 43 ± 6 μM for monoubiquitin binding to the yeast Ddi1-RVP + UBA construct (Trempe et al., 2016). The K48-linked diubiquitin showed binding to the Toxo Ddi1-RVP + UBA construct with a $K_d$ of 66 ± 5.8 μM (Fig. 8C), while it did not show binding to Cryp Ddi1-RVP.

3.4. Structure determination

Crystallization trials for three constructs of Ddi1 from *T. gondii*, Toxo Ddi1-full, Toxo Ddi1-RVP + UBA and Toxo Ddi1-RVP and three constructs of Ddi1 from *C. hominis*, Cryp Ddi1-full, Cryp Ddi1-RVP + UBA and Cryp Ddi1-RVP were carried out. Crystals were obtained for the constructs Toxo Ddi1-RVP and Cryp Ddi1-RVP. The structure of Toxo Ddi1-RVP was determined by molecular replacement using the yeast Ddi1-RVP structure (PDB Code: 2I1A) as the search model which has 45% sequence identity with Toxo Ddi1-RVP. The solution had six dimers in the asymmetric unit (ASU) (Fig. 9A) with TFZ and LLG of 35 and 3173, respectively, in the space group $P4_1212$. The following non-crystallographic symmetries relate the subunits in the ASU: (i) 2-fold symmetry that related the two halves of the ASU (Fig. 9B), (ii) 3-fold symmetry relates the three dimers on each face of the tetrahedron (Fig. 9C) and (iii) 2-fold symmetry that relates the two subunits of the dimers (Fig. 9D). Out of the six dimers in the ASU, the flap regions of both the subunits in only one dimer and the flaps of three other subunits in different dimers had electron density. The structure of Cryp Ddi1-RVP was determined by molecular replacement using the Toxo Ddi1-RVP structure as the search model which has 61% sequence identity with Toxo Ddi1-RVP. The solution had one molecule in the ASU (Fig. 9E) with TFZ and LLG of 29 and 899, respectively, in the space group $H32$. The electron density corresponding to the flap region was missing. The dimer could be generated upon application of the crystallographic 2-fold symmetry (Fig. 9F).

3.5. The structural fold

The fold and the active site architecture of Toxo Ddi1-RVP and Cryp Ddi1-RVP are similar to those of HIV protease. A structure-based sequence alignment of the RVP domains is shown in Supplementary Fig. 9.
ToxoDdi1-RVP and CrypDdi1-RVP form homodimers where each subunit is predominantly made up of β-sheets. Two aspartates, one from each subunit form the catalytic site at the dimer interface. An inter-domain β-sheet is formed by three strands from each domain (Fig. 10A).

As observed in other aspartyl protease structures, a water molecule is present within the hydrogen bonding distance of the active site aspartates in the ToxoDdi1-RVP structure (Fig. 10B).

3.6. The active site

The active site is located at the dimer interface in the cleft between the two subunits of the dimer. The motif DSG, containing the catalytic aspartate, is present at the tip of a loop called the ψ-loop in each poly-peptide chain in the active site. As observed in HIV protease and other pepsins, a network of hydrogen bonds at the dimeric interface, called the “fireman’s grip” stabilizes the active site in ToxoDdi1-RVP (Supplementary Fig. 4A) and CrypDdi1-RVP. The hydrophobic-hydrophobic-glycine (HHG) motif is a conserved structural feature of all aspartic proteases. In ToxoDdi1-RVP and CrypDdi1-RVP, it is formed by Leu-Phe-Gly. In ToxoDdi1-RVP as well as in CrypDdi1-RVP, the backbone nitrogen atoms of the catalytic aspartates are hydrogen bonded to the backbone carbonyl oxygen of the residue in the HHG motif (Supplementary Fig. 4B).

In the ToxoDdi1-RVP and CrypDdi1-RVP structures, the substrate binding groove is lined mainly by hydrophobic amino acid residues suggesting that they recognize the substrate via hydrophobic interactions. In the ToxoDdi1-RVP structure, a hydrophobic binding cavity is formed by the side chains of residues Met398, Phe305, Phe313, Ala318, Phe322, Ile353, Thr374, Leu376, Val381 and Leu384, (Supplementary Fig. 4C). Similarly in the CrypDdi1-RVP structure, the side chains of residues Met203, Tyr205, Phe218, Ala223, Ile227, Ile258, Thr279, Leu281, Val286 and Leu289 form a hydrophobic binding cavity. In both the cases, the catalytic Asp residues form an acidic patch at the centre of the cavity. The binding cavity in the ToxoDdi1-RVP structure has a width of ~27 Å (distance between the Ca atoms of Asn379 residues of the two subunits of the dimer), while in the CrypDdi1-RVP structure, the cavity width is ~22.5 Å (distance between the Ca atoms of Ser284 of the two subunits) (Supplementary Fig. 4D). The cavity in ToxoDdi1-RVP is larger than that formed in HIV protease which has a width of ~19–22 Å in the open form, while the size of the cavity in CrypDdi1-RVP is comparable to that of HIV protease.

3.7. The flap region

In HIV protease, an extended loop called the ‘flap’ covers the active site, has a β-hairpin structure and is known to stabilize the binding of the substrate or the inhibitor. The flap of ToxoDdi1-RVP forms a loop without the characteristic β-hairpin structure (Fig. 11A). The distance between the Ca atoms of Val348 residues located at the tips of the two flaps is 11.5 Å. The flaps are equidistant from the active site, as in HIV protease. The distance between the Ca atom of Val348 and the Ca atom of Asp315 (catalytic aspartate) is 21.6 Å in one subunit and 21.2 Å in the other. In the CrypDdi1-RVP structure, ten residues of the flap region could not be traced.

In HIV protease, the flap closes inwards upon substrate/inhibitor binding at the active site, assuming a closed conformation in contrast to the open or semi-open conformation in the inhibitor-free form (Fig. 11B). The two subunits were also observed to move closer to each other due to an overall conformational change. The width of the binding cavity changes from 21.9 Å (distance between the Ca atoms of Pro81 in the dimer) in its open form (PDB code: 3HVP), to 18.9 Å in its closed form.
(PDB code: 4HVP). Superposition of the structures of ToxoDdi1-RVP, LeishDdi1-RVP (PDB code: 5YQ8), yeastDdi1-RVP (PDB code: 4Z2Z) and humanDdi2-RVP (PDB code: 4RGH) reveals that the flaps of ToxoDdi1-RVP and LeishDdi1-RVP are in a more open conformation compared to those in the other two. Total conservation of half of the residues was observed when the sequences of these four flap regions were compared (Fig. 11C). Variations in the other half of the residues perhaps result in the flaps having different conformations, especially at the tips of the flaps. As a result, the width of the binding pockets varies within 5 Å in these RVP domains. This indicates that though the overall architecture of the binding pocket is retained, there are significant differences in the detailed features suggesting that the corresponding substrates can be similar but not exactly the same. To investigate the structural flexibility of ToxoDdi1-RVP and CrypDdi1-RVP, a normal mode analysis (NMA) was carried out using the elNemo server (Suhre and Sanejouand, 2004) that uses an elastic network model to generate movements in biological macromolecules that are functionally relevant. In this study, a total of 16 modes were generated and analyzed in each structure. One of the modes in each case showed significant flap movements from the open to the closed forms and movement of the entire protein to a lesser extent, leading to a change in the size of the binding cavity, as in the case of HIV protease (Fig. 12A–D).

In retropepsins, the sequence of the flap is known to play a crucial role in its dynamics and inhibitor binding. The binding affinity of HIV protease inhibitors decreased when the residues in the flap region were mutated (Yu et al., 2015). Only two glycine residues at the tip of the flap regions are conserved between the sequences of the flaps of ToxoDdi1-RVP and HIV protease. The dynamics of the flap and the nature of the substrate it binds to will depend on the sequence of the flap. An all-atom MD simulation of ToxoDdi1-RVP was carried out for 300 ns, to obtain insights into the extent of flexibility and movement of the flap region. During the simulation, the dimer remained stable and did not deviate significantly from the crystal structure. The root mean square deviation (RMSD) value of Ca atoms between the structures generated...
over the course of simulation and the crystal structure was about 3 Å. The flaps of both the subunits showed significant movement. The MD simulations showed that RMSD values of the flaps of subunits A and B stabilized at about 5.5 and 6.5 Å, respectively (Fig. 12E). This indicates a slight asymmetric behavior in the dynamics of the flaps in the dimer.

3.8. Docking of HIV protease inhibitors

Several attempts to crystallize ToxoDdi1-RVP in complex with nelfinavir and CrypDdi1-RVP in complex with saquinavir were unsuccessful. To obtain insights into possible interactions between Ddi1-RVP and inhibitors, we carried out molecular docking studies. Nelfinavir docked to ToxoDdi1-RVP with a binding energy of −7.2 kcal/mol in a manner similar to that observed in the crystal structure of the complex of HIV protease with nelfinavir. A comparison of the docking results with the crystal structure of HIV protease in complex with nelfinavir (PDB code: 2R5Q; Coman et al., 2008), reveals that the interaction between the hydroxyl group at the P1 position of nelfinavir and the carboxyl group of the active site aspartates is retained (Fig. 13A). The hydroxyl group at the P2 position of the inhibitor that interacts with the side chain of Asp29 in the case of HIV protease (Fig. 13C) interacts with the backbone –NH of Gln224 of CrypDdi1-RVP (Fig. 13D).

3.9. Binding and docking studies of the initial and the modified peptides to the Ddi1-RVP from L. major

The initial peptide (GVGRQEI) showed binding to the protein with a $K_d$ of $25.3 \pm 3.5 \mu M$ (Fig. 14A), while, the modified peptide (MIWRKPW) showed almost ten times better binding to the protein with a $K_d$ of $2.8 \pm 1.5 \mu M$ (Fig. 14B). Docking studies were performed for a better understanding of the binding modes of the initial and modified peptides to the one subunit of the ToxoDdi1-RVP dimer (Fig. 13B). Saquinavir docked to CrypDdi1-RVP with a binding energy of −6.5 kcal/mol in a similar manner as observed in the crystal structure of the complex of HIV protease with saquinavir. A comparison of the docking results of saquinavir to CrypDdi1-RVP with the crystal structure of HIV protease in complex with saquinavir (PDB code: 4QGI; Goldfarb et al., 2015), reveals that the interaction between the hydroxyl group at the P1 position of saquinavir and the carboxyl groups of the active site aspartates is retained in the docked structure. The hydroxyl group at the P2 position of the inhibitor that interacts with the side chain of Asp29 in the case of HIV protease (Fig. 13C) interacts with the backbone –NH of Gln224 of CrypDdi1-RVP (Fig. 13D).
In one of the docked conformations of the initial peptide, it was observed that it docks to the protein in an orientation similar to that observed in the crystal structure, retaining the strong ionic interaction of the Arg residue of the peptide with Asp205 and Asp205 at the active site of the protein and also the hydrogen bonding interaction of the main chain carbonyl oxygen atom of the Val residue of the peptide with the side chain of Asn210 of the protein (Fig. 15A). For the docked conformation of the initial peptide and the protein, the binding energy is $-4.5$ kcal/mol. In the docked structure of the modified peptide to LeishDdi1-RVP, it was observed that the interaction of the Arg residue to Asp205 and Asp205$'$ is retained along with the interaction of the main chain carbonyl oxygen atom of the Ile residue of the peptide to the side chain of Asn210 of the protein. In addition, another hydrogen bonding interaction between the side chain of the Trp residue of the peptide and the main chain carbonyl oxygen atom of Gly237 in the flap region of the protein was observed (Fig. 15B). The binding energy is $-6.6$ kcal/mol for the docked conformation of the modified peptide and protein. The peptides did not show any binding to ToxoDdi1-RVP and CrypDdi1-RVP as in ToxoDdi1-RVP, since the flap region makes short contacts with the initial peptide as has been observed upon superposition of ToxoDdi1-RVP on the LeishDdi1-RVP structure (Fig. 15C).
4. Discussion

HIV protease inhibitors were found to reduce the severity of protozoal infections in AIDS patients. Subsequent studies showed that the inhibitors target the Ddi1-RVP domain (White et al., 2011). More studies on the structure and dynamics of Ddi1 and its activity and inhibitor binding may direct further investigations towards the design of better antiparasitic compounds. In HIV protease, the substrate/inhibitor binding is strengthened as the subunits move closer to each other and the flap closes over the binding cavity. However, it is not clear whether the Ddi1-RVP domain behaves in a similar manner or its structural framework is too rigid to bind to HIV protease inhibitors. The present study on ToxoDdi1-RVP and CrypDdi1-RVP domains aims to address these points. The crystal structures of ToxoDdi1-RVP and CrypDdi1-RVP show that they share several structural features such as the fold, the active site architecture, and the hydrophobic nature of the substrate binding cavity, with HIV protease. The binding cavities of ToxoDdi1-RVP, yeastDdi1-RVP (Sirkis et al., 2006) and LeishDdi1-RVP (Kumar and Suguna, 2018) are similar in size but larger compared to that of HIV protease. In addition, there are variations in the binding site residues. Instead of Asp29, Asp30, and Val82, which interact with inhibitors in HIV protease, ToxoDdi1-RVP has Gln319, Ser320, and Glu382, and CrypDdi1-RVP has Gln224, Thr225, and Asp287, respectively. Due to these small but subtle variations, the nature of the inhibitors may be different as the surface charge and polarity of the binding site are altered. Further, contrary to the case in HIV protease, the flap of ToxoDdi1-RVP has no secondary structure. The flap regions in Ddi1-RVP appear to be highly mobile as is evident from the missing electron density for one of the flaps in yeastDdi1-RVP and humanDdi2-RVP and for both the flaps in CrypDdi1-RVP. The electron density for the flaps of both the subunits of one dimer is visible in ToxoDdi1-RVP. The larger binding cavity and the presence of such a flexible flap suggest that Ddi1-RVP may accommodate bigger substrates. Nrf1 was proposed to be the natural substrate of human Ddi2 (Koizumi et al., 2016); however, protozoa do not have any homologues of Nrf1, implicating that protozoal Ddi1 will target entirely different molecules. Further, the NMA analysis that we carried out to explore possible conformational changes and modulation of the binding pocket due to inhibitor binding revealed the structural flexibility of ToxoDdi1-RVP and CrypDdi1-RVP dimers similar to that of HIV protease. The binding affinities of ToxoDdi1-RVP and CrypDdi1-RVP for HIV protease inhibitors are in the micromolar range (compared to nanomolar values for HIV protease) in solution as suggested by BLI studies. The MD simulation studies carried out on ToxoDdi1-RVP reveal that the flaps are highly flexible. Also, the flaps of the two subunits of the dimer show a slightly asymmetric behaviour in terms of their dynamics. The peptide inhibitor proposed from the crystal structure of LeishDdi1-RVP shows binding to the protein through BLI studies and the modified peptide generated shows better binding than the initial peptide as indicated by their $K_d$ values.

Our study demonstrates the proteolytic activity of the isolated Ddi1-RVP domain for the first time. YeastDdi1 was shown to act on polyubiquitinated (more than eight chains) substrates (Yip et al., 2020) but the cleavage of peptides by neither the full length Ddi1 (Trempe et al., 2016) nor the isolated RVP domain was detected. Both the UBL and the HDD domains were found to be essential for the binding of the substrates. No activity was detected for humanDdi2 or its RVP domain (Sivá et al., 2016). Proteolytic activity (in vitro) was detected only for LeishDdi1-fl using the synthetic HIV protease peptide substrate-1 at pH 5 (Perteguer et al., 2013). While the presence of HDD and the similarities in the structures and the active site architecture suggest that ToxoDdi1-RVP may have the capability to cleave ubiquitin and the substrates upon polyubiquitination similar to the yeastDdi1, in the case of CrypDdi1-RVP as the HDD domain is missing, the triggering mechanism has to be different and is yet to be investigated. Subtle but significant changes in the geometry and residues in the substrate binding cavities of the RVP domains and marked differences in the domain organization of the full length proteins in different organisms suggest that the corresponding substrates and/or cleavage sites could vary considerably while sharing some common features.

For a long time after protection against protozoal infections by HIV protease inhibitors was detected, efforts for the development of more effective drugs did not progress well, as the target for these inhibitors was not known. Though Ddi1 was later identified as a possible target, there is a dearth of structural information on Ddi1. The present study on the crystal structures of the RVP domains of ToxoDdi1 and CrypDdi1 contributes to expand the available structural data base on these proteins. Though HIV protease inhibitors showed weak binding, it is an encouraging result and provides a starting point for further improvements and an alternate strategy to the pursuit to develop specific inhibitors to Ddi1 in protozoa.
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**CRediT authorship contribution statement**

**Killivalavan Asaithambi**: Formal analysis, Writing – original draft.  
**Iman Biswas**: Formal analysis, Writing – original draft.  
**Kaza Suguna**: Formal analysis, Writing – original draft.

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**Fig. 15.** Active site interactions of LeishDdi1-RVP (PDB code: 5YQ8, Kumar and Suguna, 2018) with peptide inhibitors. (A) Initial peptide (GVGRQE) (B) Modified peptide (MIWRKW) and (C) Superposition of ToxoDdi1-RVP (orange) on LeishDdi1-RVP (purple) with the initial peptide. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crstbi.2022.05.003.

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