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Feline infectious peritonitis (FIP) was first described in 1963 and was initially termed ‘chronic fibrinous peritonitis’.1 Today, FIP is a deadly disease that affects both domestic and wild cats and is caused by a mutation in feline coronavirus (FCoV) that allows the virus to replicate in macrophages. Currently, there are no treatments or vaccines available for the treatment of FIP even though it kills approximately 5% of cats in multi-cat households per year. In an effort to develop small molecule drugs targeting FIP for the treatment of cats, we screened a small set of designed peptidomimetic inhibitors for inhibition of FIPV-3CLpro, identifying two compounds with low to sub-micromolar inhibition, compound 6 ($IC_{50} = 0.59 \pm 0.06 \mu M$) and compound 7 ($IC_{50} = 1.3 \pm 0.1 \mu M$). We determined the first X-ray crystal structure of FIPV-3CLpro in complex with the best inhibitor identified, compound 6, to a resolution of 2.10 Å to better understand the structural basis for inhibitor specificity. Our study provides important insights into the structural requirements for the inhibition of FIPV-3CLpro by peptidomimetic inhibitors and expands the current structural knowledge of coronaviral 3CLpro architecture.

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| Compd | Structure | %I (50 μM) | %I (10 μM) | %I (5 μM) |
|-------|-----------|------------|------------|----------|
| 1     | ![Structure 1](image1.png) | 54.8 ± 5.7 | 36.4 ± 0.4 | 30.1 ± 1.9 |
| 2     | ![Structure 2](image2.png) | 39.2 ± 0.5 | 12.8 ± 2.2 | 15.6 ± 0.8 |
| 3     | ![Structure 3](image3.png) | 52.3 ± 1.4 | 36.0 ± 3.9 | 31.3 ± 1.1 |
| 4     | ![Structure 4](image4.png) | 55.6 ± 1.9 | 34.4 ± 2.9 | 32.7 ± 1.3 |
| 5     | ![Structure 5](image5.png) | 17.6 ± 1.2 | 11.2 ± 3.4 | 2.9 ± 2.7 |
| 6     | ![Structure 6](image6.png) | 99.7 ± 0.2 | 92.1 ± 4.3 | 89.8 ± 2.1 |
| 7     | ![Structure 7](image7.png) | 99.4 ± 0.02 | 92.2 ± 1.4 | 85.8 ± 4.9 |
| 8     | ![Structure 8](image8.png) | 94.7 ± 1.3 | 69.1 ± 1.7 | 53.7 ± 4.0 |
| 9     | ![Structure 9](image9.png) | 82.5 ± 1.8 | 33.3 ± 0.2 | 26.9 ± 0.5 |

(continued on next page)
cysteine (Cys144) of FIPV-3CLpro, and they are similar to other previously reported peptidyl inhibitors of various 3CLpros.16–19 The synthesis of the eleven tested peptidomimetics in Table 1 has been previously reported.15,20

Compounds (1–11) were initially tested at three concentrations (5 μM, 10 μM, and 50 μM) to identify inhibitors of FIPV-3CLpro and the results are reported in Table 1. A trend is immediately observed in the six compounds with the highest inhibition of FIPV-3CLpro (6–11), that is, having greater than 60% inhibition at a concentration of 50 μM, where a leucine residue is favored in the P2 position of the inhibitor over phenyl or isopentenyl groups (1–3). Analysis of the eleven cleavage sites within the FIPV replicase polyprotein 1ab

Figure 1. FIPV-3CLpro cleavage sites in polyprotein 1ab and inhibition by compounds 6 and 7. (A) The eleven polyprotein 1ab recognition sequences for FIPV-3CLpro are shown from P5 to P4′ under the blue shaded box in their respective binding locations. FIPV-3CLpro is represented by the blue shaded box where the subsites are represented as pockets and labeled accordingly. Subsites with no clear residue preferences are outlined by a dashed line, indicating they may not exist. Peptidomimetic inhibitor 6 (P4 = S) or 7 (P4 = T) binds to FIPV-3CLpro via nucleophilic attack of Cys144 at the β-carbon of the α,β-unsaturated ethyl ester, where the pyrrolidinonyl methyl acts as the P1 residue, Leu as

Table 1

| Compd | Structure | %I (50 μM)  | %I (10 μM)  | %I (5 μM)  |
|-------|-----------|-------------|-------------|-------------|
| 10    | ![Structure](image1) | 97.0 ± 0.9  | 58.6 ± 3.9  | 37.0 ± 2.4  |
| 11    | ![Structure](image2) | 66.3 ± 0.2  | 20.5 ± 1.9  | 13.4 ± 3.7  |

Figure 1. FIPV-3CLpro cleavage sites in polyprotein 1ab and inhibition by compounds 6 and 7. (A) The eleven polyprotein 1ab recognition sequences for FIPV-3CLpro are shown from P5 to P4′ under the blue shaded box in their respective binding locations. FIPV-3CLpro is represented by the blue shaded box where the subsites are represented as pockets and labeled accordingly. Subsites with no clear residue preferences are outlined by a dashed line, indicating they may not exist. Peptidomimetic inhibitor 6 (P4 = S) or 7 (P4 = T) binds to FIPV-3CLpro via nucleophilic attack of Cys144 at the β-carbon of the α,β-unsaturated ethyl ester, where the pyrrolidinonyl methyl acts as the P1 residue, Leu as
shows that nine of the eleven cleavage sites have a leucine residue at the P2 position and none have a phenylalanine residue (Fig. 1A), which is the P2 residue that is present in compounds 1, 2, 4, 5, and 21. Furthermore, the two best peptidomimetic inhibitors, compounds 6 and 7, have either a serine or threonine residue at the P4 position. A P4 serine is present in two of the eleven FIPV-3CLpro cleavage sites, while a P4 threonine is present in four of the eleven cleavage sites (Fig. 1A).

Comparison of compounds 6 and 10, which are only different at the N-termini where 6 has a tert-butyl carbamate and 10 has a bis-tetrahydrofuranyl carbamate, shows that the increase in steric bulk associated with the N-terminus of 10 decreases FIPV-3CLpro inhibition at every concentration of 10. Furthermore, comparison of 10 to 11, which are only different in that 10 has a free P4 serine while 11 has a P4 bis-tetrahydrofuranyl carbonate, shows that the increase in steric bulk at P4 of 11 decreases FIPV-3CLpro inhibition at every concentration of 11. Comparison of 7 to 8, which are only different in that 7 has a P4 threonine and 8 has a P4 L-allo-threonine, shows that the stereochemistry of the P4 residue is important for good FIPV-3CLpro inhibition, where 7 has increased inhibition of FIPV-3CLpro at all inhibitor concentrations tested.

We then determined the IC50 values for the two best peptidomimetic inhibitors of FIPV-3CLpro, 6 and 7. These compounds were chosen because they are the only compounds in the series that have greater than 80% inhibition of FIPV-3CLpro at a concentration of 5 μM. The IC50 values of 6 and 7 were determined after pre-incubation of FIPV-3CLpro with the respective peptidomimetic inhibitor at both 15 and 30 min. We found compound 6, which showed slightly better inhibition of FIPV-3CLpro than compound 7, to have an IC50 of 0.69 ± 0.07 μM after 15 min of pre-incubation and an IC50 of 0.59 ± 0.06 μM after 30 min of pre-incubation. We found 7 to have an IC50 of 1.7 ± 0.2 μM after 15 min of pre-incubation and 1.3 ± 0.1 μM after 30 min of pre-incubation (Fig. 1B).

To gain a more complete structural understanding of how the peptidomimetic compounds bind to and inhibit FIPV-3CLpro, we crystallized and determined the X-ray structure of FIPV-3CLpro bound to compound 6 via a covalent bond with the active site cysteine (Cys144). The statistics for X-ray data collection, processing, and refinement are given in the Supplementary material.22–24 The X-ray structure of the FIPV-3CLpro:6 complex was determined to 2.1 Å resolution (Rint = 4.0%, Rwork = 17.6% Rfree = 23.0%) with four monomers in the asymmetric unit (Fig. 2A and B). The four monomers in the asymmetric unit represent two complete,
biologically active dimers of the FIPV-3CL\(^{\text{pro}}\)-6 complex. Electron density associated with compound 6 is clearly visible in final, \(F_o - F_e\) electron density omit maps contoured to +3.0\(\sigma\) (Fig. 2C).

Compound 6 binds in the active site of FIPV-3CL\(^{\text{pro}}\), mimicking the native polyprotein substrate (Figs. 2 and 3). The pyrrolidinonyl methyl acts as the \(P_1\) residue mimicking glutamine in the polyprotein, while the \(\beta\)-carbon of the \(\alpha\)-\(\beta\)-unsaturated ethyl ester is covalently bonded (distance of 1.9 Å) to the sulfur atom of the catalytic cysteine, Cys144 (Figs. 2B and 3). The leucine residue of 6 acts as the \(P_2\) residue and occupies the \(S_2\) subsite pocket, whereas the valine and serine residues act as the \(P_3\) and \(P_4\) residues. The terminal tert-butyl carbamate group extends to the edge of the FIPV-3CL\(^{\text{pro}}\) active site cleft (Figs. 2C and 3).

Compound 6 forms eight direct hydrogen bonds to five active site residues of FIPV-3CL\(^{\text{pro}}\). In addition, two water-mediated hydrogen bonds are formed between compound 6 and backbone carbonyl oxygens of Thr47 and Ser48 (Fig. 3). The backbone amide –NH of Gly142 forms a 2.7 Å hydrogen bond to the carbonyl of the ethyl ester, which sits in the \(S_1\) pocket of FIPV-3CL\(^{\text{pro}}\). The pyrrolidinonyl methyl of 6 is anchored into the \(S_2\) subsite pocket by two hydrogen bonds, a 2.7 Å hydrogen bond from the side-chain tel-e-nitrogen of His162 to the lactam carbonyl, and a 3.0 Å hydrogen bond from the lactam –NH to the side chain carbonylate of Glu165. Each of the four backbone NHs of 6 forms a hydrogen-bond to an FIPV-3CL\(^{\text{pro}}\) active site residue—three direct hydrogen bonds and one water-mediated hydrogen bond. Two of the four backbone carbonyls of 6 participate in hydrogen-bonding to FIPV-3CL\(^{\text{pro}}\) active site residues—a direct hydrogen bond from the backbone –NH of Glu165 and a water-mediated hydrogen-bond to the backbone carbonyls of Thr47 and Ser48 (Fig. 3).

Examination of the FIPV-3CL\(^{\text{pro}}\) \(S_2\) pocket shows that it is large enough to accommodate a \(P_2\) leucine residue, but may not be sufficiently large enough to allow optimal binding of peptidomimetic inhibitors with larger \(P_2\) groups, such as phenylalanine or larger alkyl groups, which introduce more steric bulk (i.e. inhibitors 1–5, Table 1). Interestingly, Kim and coworkers have shown that peptidomimetic dimers, similar to our compounds 1–3, with sterically bulky \(P_2\) substituents (leucine, benzyl or cyclohexyl-methyl) are effective against FCoV in cell-based antiviral assays.\(^{18}\) This suggests that despite the reduced inhibition of FIPV-3CL\(^{\text{pro}}\) by compounds 1–5 relative to 6–11, these compounds may be effective in cell-based antiviral assays. Kim et al. did not test their compounds against purified FIPV 3CL\(^{\text{pro}}\) enzyme suggesting that our compounds 6–11 may be even more potent in FCoV antiviral assays. Furthermore, Kim et al. have demonstrated that peptidomimetics akin to 1–3 and 5–11 are capable of reducing viral titers and pathological lesions in the livers of mice infected with murine hepatitis virus (MHV), a coronavirus similar to FCoV.\(^{18}\) Together, our studies and those of Kim et al. suggest that peptidomimetic compounds like 1–3 and 6–11 may have significant potential for being developed into effective therapeutics against FIPV infection.

In conclusion, we have over-expressed and purified FIPV-3CL\(^{\text{pro}}\) and determined its inhibition by a set of eleven peptidomimetic inhibitors. We found that the six best inhibitors of FIPV-3CL\(^{\text{pro}}\) (6–11) had a leucine residue at the \(P_2\) position and a valine residue at the \(P_3\) position, which is consistent with these residues being preferred at the \(P_2\) and \(P_3\) positions of the polyprotein substrate (Fig. 1A). We determined the X-ray structure of FIPV-3CL\(^{\text{pro}}\) in complex with the best peptidomimetic inhibitor identified, compound 6, which is the first reported structure of FIPV-3CL\(^{\text{pro}}\). The molecular details elucidated from the X-ray structure of FIPV-3CL\(^{\text{pro}}\) in complex with 6 provide insights into the key interactions between the inhibitor and the enzyme that can be targeted in the design of more potent FIPV-3CL\(^{\text{pro}}\) inhibitors.

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Supplementary data

Supplementary data (materials and methods and X-ray data-collection and refinement statistics for FIPV-3CLpro:6 complex (PDB ID = 4ZRO)) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.10.023.

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