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Design and synthesis of irreversible inhibitors of foot-and-mouth disease virus 3C protease

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ABSTRACT

Foot-and-mouth disease virus (FMDV) causes a highly infectious and economically devastating disease of livestock. The FMDV genome is translated as a single polypeptide precursor that is cleaved into functional proteins predominantly by the highly conserved viral 3C protease, making this enzyme an attractive target for antiviral drugs. A peptide corresponding to an optimal substrate has been modified at the C-terminus, by the addition of a warhead, to produce irreversible inhibitors that react as Michael acceptors with the enzyme active site. Further investigation highlighted key structural determinants for inhibition, with a positively charged P2 being particularly important for potency.

Foot-and-mouth-disease virus (FMDV) is the causative agent of a highly contagious disease of cloven-hoofed mammals and disease outbreaks have significant economic impact.[1] It is generally associated with low levels of development and it has become enzootic in areas of Africa, Asia, South America and the Middle East. However, it also occurs sporadically in areas that are usually free of the disease, most notably recently in the United Kingdom in 2001[2] and 2007[3] and in Japan in 2010.[4]

Although use of FMDV vaccines has been successful in reducing the frequency of outbreaks,[5] preventive vaccination is not practiced in the United States or Europe due to the restrictions it entails on international trade of animals and animal products and the inability to detect carriers in vaccinated populations.[6] An additional hazard of vaccination is that FMDV vaccine production utilises live virus, which presents a containment risk. There is evidence that some FMD outbreaks have actually had a vaccine origin due to incomplete inactivation prior to formulation,[7] or result from the escape of the virus from laboratory sources.[8] These events highlight the need for more effective control methods and, in fact, vaccines that do not require infectious FMDV at any stage of their production are in development.[9] An alternative strategy is the design of anti-FMDV drugs.

FMDV belongs to the *Aphtovirus* genus of the *Picornaviridae* family and has a small non-enveloped icosahedral capsid that contains a linear, positive sense RNA genome.[10] This highly contagious viral family includes human rhinovirus (HRV), hepatitis A virus (HAV) and poliovirus (PV). The molecular basis of FMDV pathogenesis and the regulation of picornavirus gene expression have been reviewed.[11] A key step in viral replication is the cleavage of a single polyprotein to form the final mature structural and functional viral proteins. A total of 13 cleavages are required, of which 10 are performed by one of the virally-encoded proteins, 3C protease ($3C^{pro}$). This makes the FMDV $3C^{pro}$ enzyme a potential drug target for preventing viral replication, with no known cellular homologues being found in susceptible hosts. The structures of $3C^{pro}$ enzymes from the related HRV,[12] HAV[13] and PV[14] have been determined, and we have solved the structure of FMDV $3C^{pro}$,[15] which may assist this process. Structurally, the enzyme is found to be a chymotrypsin-like cysteine protease.[13,15a,c]

Furthermore, FMDV $3C^{pro}$ is one of the most highly conserved proteins in the viral genome, being 76% identical in amino acid sequence across all serotypes,[16] with the substrate-binding site being one of the most highly conserved features of the enzyme. Protease inhibitors may therefore have the advantage of being active against a range of different FMDV serotypes, in marked contrast to vaccines that are serotype-specific.

The $3C^{pro}$ from HRV has been the subject of an extensive study to find inhibitors, with peptidic α,β-unsaturated esters being found to act as selective mechanism-based inhibitors.[17] These inhibitors add a Michael acceptor warhead on to a short peptide that represents the preferred P-side substrate sequence for the enzyme (for

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an overview see Ref. 18). This route has led to some potent HRV $3C^{pro}$ inhibitors: Ruprintrivir (AG7088) is an irreversible inhibitor with low nM EC_{50} values.[19] Although initial proof of concept was achieved,[20] Rupintrivir did not significantly enhance recovery levels during a natural infection study and development was stopped.[21] Nonetheless, structural aspects of Rupintrivir have been used in efforts to inhibit various 3C-like viral cysteine proteases.[22]

These efforts have focused on the design of broad spectrum anitivirals based on the similarities between such viral proteases. For example the structural relationship between FMDV $3C^{pro}$ and HRV $3C^{pro}$ allows the potent inhibition of the former by Rupintrivir, designed for the latter.[22c] In contrast, the work presented here uses structural information from our previous studies to design an inhibitor specific to FMDV $3C^{pro}$. The optimal substrate for FMDV $3C^{pro}$ has the sequence APAKQ-LLNFD, which corresponds to the VP1/2A junction of the viral polyprotein.[15a,c] Of this sequence, the P4-P1 residues (PAKQ) are the most critical for selectivity. The concept is illustrated in Scheme 1, yielding compound **1** as a potential inhibitor.

Two different synthetic routes were used to make the inhibitors described in the present study, as illustrated below for the synthesis of inhibitor **1**. In both cases, the unsaturated ester moiety was achieved by a Wittig or Horner–Wadsworth–Emmons (HWE) reaction and the peptide sequence through solid phase peptide synthesis (SPPS). In one route (Scheme 2), glutamine is loaded onto Wang resin and SPPS carried out. The aldehyde **2** is then achieved by reductive cleavage of the C-terminus of the peptide from the resin. An HWE reaction to form the Michael Acceptor **3**, followed by deprotection of the side chains completes the synthesis. The other route (Scheme 3) utilises a Wittig reaction to pre-form a building block onto which the peptide sequence is subsequently assembled. A protected glutamic acid **4** was first modified on its C-terminus to provide the corresponding Michael acceptor **5**.[23] The acid can then be attached to resin and the peptide sequence added on as before.

Additionally, the N terminus of the peptide can be acetylated prior to deprotection and cleavage.

Both routes offer advantages and disadvantages. Route A is a more convenient way to generate a range of different warheads having the same peptide sequence. Route B is better suited to production of peptide variants of a particular Michael acceptor warhead. The latter route is also dependent on attaching the modified amino acid via the side chain; it is therefore useful for glutamine variants (as here) and with slight modification could yield asparagine, glutamate or aspartate sequences. A minor limitation of route B is that the warhead needs to be resistant to the peptide synthesis conditions (particularly the TFA cleavage). An advantage is that although this synthetic route is slightly longer than route A the overall yield is much higher (on average 37% after 15 steps compared with 3–6% over 13 steps).

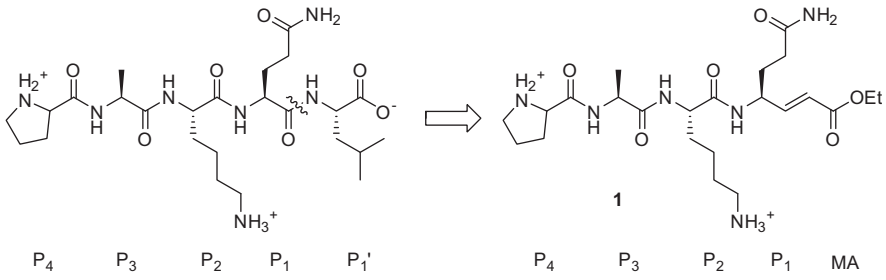
To characterise the compounds synthesised were tested for their ability to inhibit recombinant FMDV $3C^{pro}$ in vitro in the presence of the previously described fluorogenic substrate *DABCYL*-APAKQ↓LLD(*EDANS*)FDLLK, where ↓ marks the position of the scissile bond.[24,25]

Initially, 4 analogues of the inhibitor were synthesized, corresponding to the various lengths of P-side peptide sequence (Table 1). It was found that sequences which included up to P5 (**6**) or P4 (7) acted as potent inhibitors of the enzyme with apparent IC_{50} values in the sub-micromolar range; compound **8**, which extends only to P3 was a significantly poorer inhibitor while compound **9**, which extends only to the P2 position, is essentially inactive as an inhibitor. These data are consistent with the substrate specificity studies of FMDV $3C^{pro}$, which show that sequence changes at P4, P2 and P1 result in marked loss of activity.[15a,c] The presence or absence of a P5 residue was found to have little influence on activity in this assay. However, it is known that that residues with very different structures (Ala, Pro, Arg, Asp, Glu and Leu) are found among the naturally occurring cleavage sequences of FMDV $3C^{pro}$ at P5,[26] suggesting that few if any specific interactions are made with this side chain.

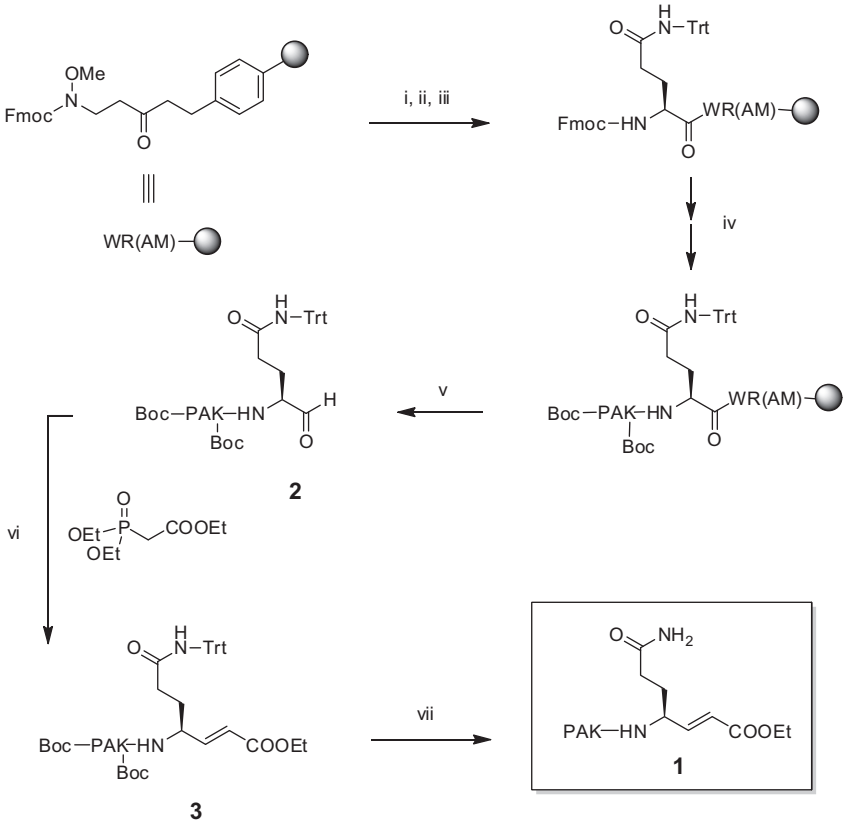
It should be noted that the assays in this study were performed at an enzyme concentration of 0.6 μM, which was necessary in order to determine the rate of substrate hydrolysis (the activity of this viral protease is far lower than typical digestive proteases, necessitating high enzyme concentrations for reliable assay data). As IC_{50} cannot be less than 50% enzyme concentration this sets a lower threshold for what can be observed in these assays. It is therefore possible that the measured IC_{50} values for the best inhibitors are underestimates of the potency at lower enzyme concentration.

Initially, all compounds were *N*-acetylated. To examine the effect of N-terminal modification on inhibitory potency, analogues of compound **7** were synthesized (Table 2). Compounds **10**, **11** and **12** were generated by capping the tetrapeptide with benzoyl, tosyl and benzyl respectively and **13** by capping the tripeptide with benzoyl. Compound **1**, which lacks the N-terminal acetylation, was found to be a poorer inhibitor than compound **7**. This suggests that the presence of a charged N-terminus is sub-optimal for inhibition. The natural substrates for $3C^{pro}$ are extended polypeptides, which the *N*-acylated version is expected to mimic more closely. All *N*-capped versions of compound **1** (**7**, **10**, **11**, **12**) were found to have improved activity, reinforcing this view. The best inhibitor of the series has a benzoyl cap (**12**). A benzoyl cap is also found to improve inhibition when the P4 Pro residue is removed (**13**); this shorter peptide is more active than the corresponding acetylated version (**8**) although loss of the P4 Pro results in less potent inhibition overall.

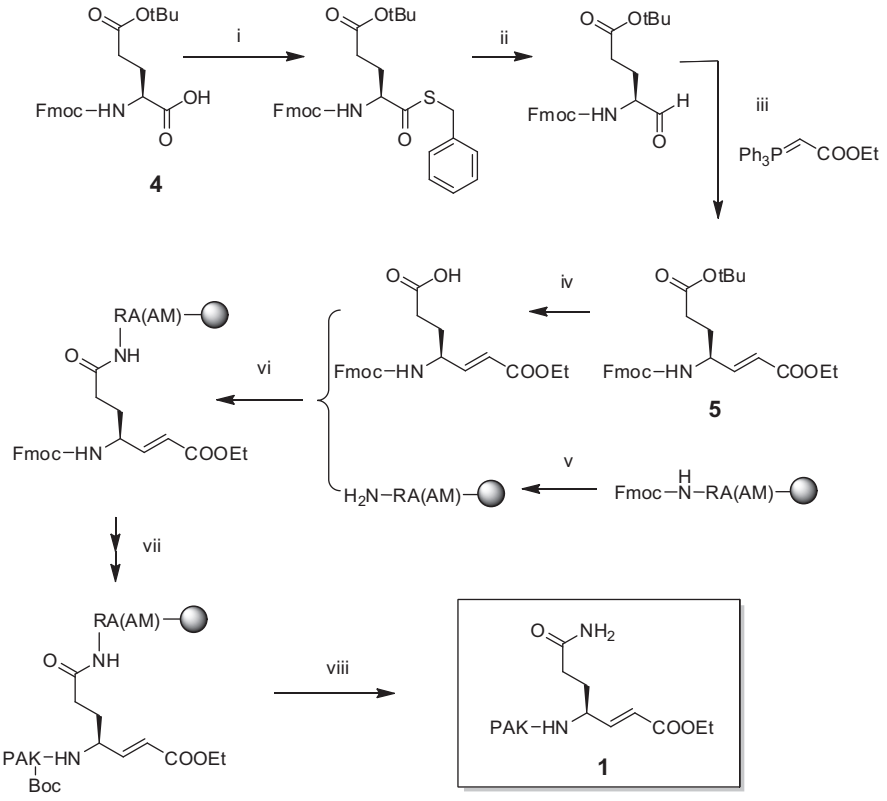
In the co-crystal structure of the enzyme with a peptide substrate, the P5 position is mostly solvent exposed and lacks significant enzyme contacts,[15c] which is illustrated in Figure 1. This is consistent with P5 variations having only minor effects on inhibitor potency.



Scheme 1. FMDV $3C^{pro}$ P4–P1′ substrate sequence and the first FMDV $3C^{pro}$ inhibitor design (**1**). A tetrapeptide is modified on its N-terminus containing a Michael acceptor (MA) moiety, in this case an α,β-unsaturated ester, taking the P1′ position of the original substrate.

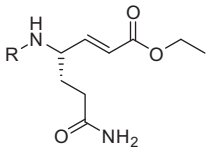


Scheme 2. Synthesis of Michael acceptor peptides (route A). Reagents and conditions: (i) 20% piperidine in DMF. (ii) DIPCDI, HOAt, FmocGln(Trt)OH. (iii) Ac_20, Dipea. (iv) Fmoc SPPS. (v) $LiAlH_4$ in THF, −30 °C. (vi) NaHMDS in THF, −78 °C. (vii) TFA/H_2O/TIS, 95:2.5:2.5. 6% yield.



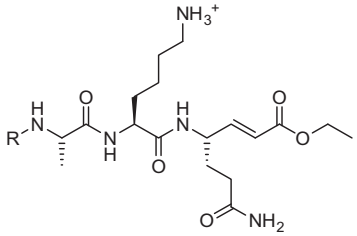
Scheme 3. Synthesis of Michael acceptor peptides (route B). Reagents and conditions: (i) DCC/DNAP, BnSH. (ii) Et_3SiH, Pd/C. (iii) THF (iv) 10% TFA in DCM. (v) 20% piperidine in DMF. (vi) HOAt/HATU/DIPEA. (vii) Fmoc SPPS. (viii) TFA/H_2O/TIS, 95:2.5:2.5. 37% yield.

Table 1
Effect of peptide chain length on antiprotease activity against FMDV $3C^{pro}$



Compound	–R	IC_{50} (μM)
6	Ac-Ala-Pro-Ala-Lys–	0.64 ± 0.08
7	Ac-Pro-Ala-Lys–	0.68 ± 0.06
8	Ac-Ala-Lys–	17 ± 0.8
9	Ac-Lys–	>600

Table 2
Effect of N-terminal cap on antiprotease activity against FMDV $3C^{pro}$



Compound	–R	IC_{50} (μM)
1	H-Pro–	4.6 ± 0.03
7	Ac-Pro–	0.68 ± 0.06
10	Bz-Pro–	0.43 ± 0.03
11	Tos-Pro–	1.6 ± 0.1
12	Bn-Pro–	1.5 ± 0.1
8	Ac–	17 ± 0.8
13	Bz–	2.3 ± 0.3

The nature of the electron withdrawing group within the Michael acceptor warhead is known to modulate the potency of inhibitors of human rhinovirus $3C^{pro}$.[17] Inhibitors with three different EWGs were compared for inhibition potency, these being the ethyl ester (**7**), benzyl ester (**14**) and *N*-methoxy-*N*-methyl carboxamide (**15**) analogues (Table 3). The most potent warhead was the α,β-unsaturated ethyl ester, as indeed was the case for the rhinovirus enzyme. The other two analogues tested were less potent, with the *N*-methoxy-*N*-methyl carboxamide having an IC_{50} value 100-fold higher than the ethyl ester. At present it is not clear why the ester group has such a large effect on activity but these results show that variation in this position does have a significant effect on potency. In particular, there is no structural information that could be used to explain these differences and it is likely that a more extensive set of variants would reveal more potent analogues.

We have previously shown that a lysine residue is preferred at the P2 position of FMDV $3C^{pro}$ substrates.[15a] Substrates containing other positively charged residues (Orn and Arg) are also hydrolysed by the protease whereas the presence of neutral side chains at P2 (Thr and Nle) abrogated hydrolysis.[15c] The crystal structure of $3C^{pro}$ with bound peptide substrate reveals that the preference for a P2 Lys side chain is due to salt-bridge interactions with two aspartic acid residues (Asp144 and Asp146) in the flexible β-ribbon of the enzyme[15c] as shown in Figure 1.

To examine the role of P2, a series of compounds containing a range of P2 substitutions was synthesised and compared to the

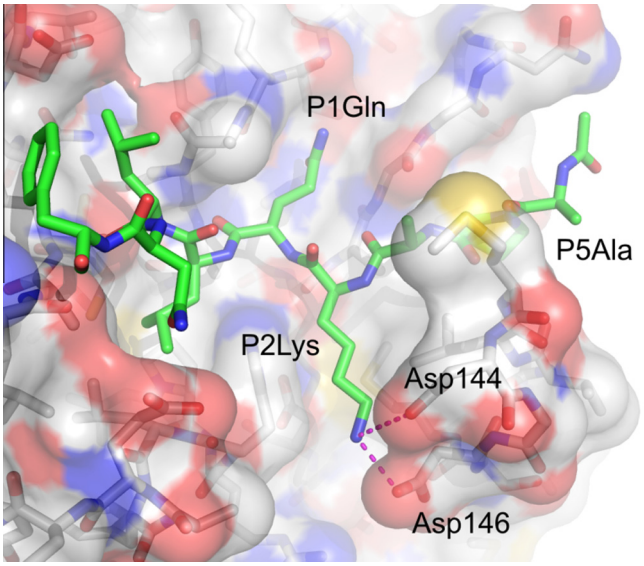
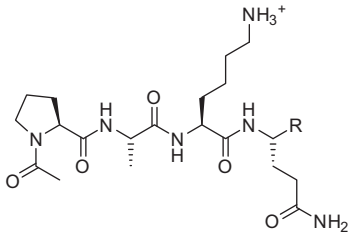
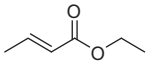
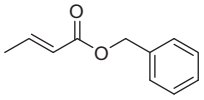
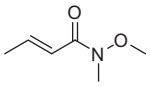


Figure 1. The substrate binding pocket of FMDV $3C^{pro}$. The structure of a substrate peptide bound into the active site of $3C^{pro}$, illustrating that the P5 residue is largely solvent exposed and that a P2 Lys residue makes interactions with the side chains of two Asp residues within the active site.

Table 3
Effect of Michael acceptor variation on antiprotease activity against FMDV $3C^{pro}$

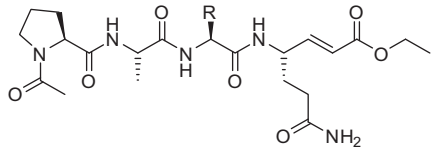


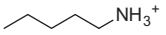
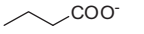
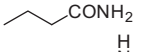
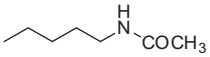
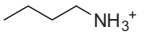
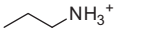
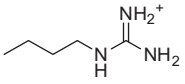
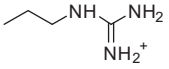
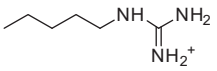
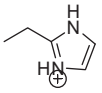
Compound	–R	IC_{50} (μM)
7		0.68 ± 0.06
14		7.7 ± 0.7
15		66 ± 6

P2 lysine variant **7** (Table 4). These inhibitors include variants in which the positive charge was deleted, replaced by a negative charge or subtly relocated using other positively-charged analogues. In general, the P2 preference for the inhibitors mirrors that for substrates, with positively charged residues being greatly preferred. Lys is the optimal residue, but Arg is almost as potent and Orn and His also provide significant inhibition. In contrast, an anionic residue is deleterious to inhibition, **17** giving no inhibition even when at 500 μM.

To obtain evidence for the formation of an irreversible complex, compound **7** was incubated with the FMDV $3C^{pro}$ enzyme and the inhibited complex was analysed by electrospray mass spectroscopy. Mass analysis of FMDV $3C^{pro}$ revealed a mixture of species with molecular weights 23,106, 23,182 and 23,258 Da. The expected molecular weight is 23,032 Da. We interpret these data as indicative of the presence of β-mercaptoethanol adducts,[27] where

Table 4
Specificity of P2 sequence for antiprotease activity against FMDV $3C^{pro}$



Compound	P2	–R	IC_{50} (μM)
7	Lys		0.68 ± 0.06
16	Gly		2800 ± 445
17	Glu		NI[†]
18	Gln		53 ± 0.2
19	Lys(Ac)		130 ± 12
20	Orn		2.7 ± 0.1
21	Dab		21 ± 0.8
22	Arg		0.84 ± 0.03
23	nArg		3.8 ± 0.1
24	hArg		1.2 ± 0.2
25	His		9.8 ± 0.3

[†] NI = No inhibition observed at the highest inhibitor concentration tested (500 μM).

1, 2 or 3 molecules of mercaptoethanol (76 Da) remain associated from the purification buffers. After incubation with compound **7**, the most abundant peak in the mass spectrum corresponded to a mass of 23,640 Da. The addition of one molecule of inhibitor would increase the mass of the enzyme by 538 Da; the experimentally determined increase of 534 Da compared with the lowest mass found in the native enzyme is consistent with this, within the error limits of the mass determination in this experiment (roughly ± 4 Da). It is therefore found that the inhibited enzyme shows the attachment of a single molecule of inhibitor, providing good evidence for the desired mode of inhibition.

In summary, we have shown that peptidic analogues of an optimal $3C^{pro}$ cleavage sequence that contain a Michael acceptor warhead act as potent inhibitors of this enzyme. The mechanism of inhibition involves the formation of a covalent 1:1 complex with the enzyme, which we presume to be via reaction with the catalytic cysteine residue. Potent inhibition requires a four-residue peptide sequence and a blocked N-terminus is preferred. Inhibition potency strongly mirrors substrate specificity, with a marked preference for a positively charged residue in the P2 position. The nature of the EWG that forms part of the Michael acceptor also strongly influences reactivity, with an ethyl ester being the most potent of those tested.

Acknowledgments

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