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P_1 Glutamine isosteres in the design of inhibitors of 3C/3CL protease of human viruses of the *Pisoniviricetes* class

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Viral infections are one of the leading causes of acute morbidity in humans and much endeavour has been made by the synthetic community for the development of drugs to treat associated diseases. Peptide-based enzyme inhibitors, usually short sequences of three or four residues, are one of the classes of compounds currently under development for enhancement of their activity and pharmaceutical properties. This review reports the advances made in the design of inhibitors targeting the family of highly conserved viral proteases 3C/3CL^{pro}, which play a key role in viral replication and present minimal homology with mammalian proteases. Particular focus is put on the reported development of P_1 glutamine isosteres to generate potent inhibitors mimicking the natural substrate sequence at the site of recognition.'

Introduction

The *Pisoniviricetes* class encompasses a diverse range of positive-sense single-stranded RNA viruses infecting eukaryotic organisms. Importantly to humans, this class includes the *Picornaviridae*, *Coronaviridae* and *Caliciviridae* families which represent some of the leading causes of acute morbidity in humans,[1] and are among the most prevalent infectious agents. Picornaviruses are a large family of viruses, infecting both humans and animals, and their pathology ranges from mild infections such as the common cold and hepatitis A to more severe disease including meningitis and paralysis. In some instances viral infections by picornaviruses have been linked to autoimmune diseases such as myocarditis, diabetes, and multiple sclerosis.[2–6] Coronavirus infections also span a range of severity,[2,7] while caliciviruses are the leading cause of acute gastroenteritis but can have more severe symptoms in immunocompromised individuals.[8] One of the unifying features of the *Pisoniviricetes* class is a highly structurally conserved cysteine protease belonging to the PA clan (proteases of mixed nucleophile, superfamily A), commonly referred to as 3C^{pro} (picornaviruses) or 3CL^{pro} (coronaviruses and caliciviruses, also known as M^{pro}). 3C^{pro} and 3CL^{pro} enzymes play a critical role in viral replication, initially catalysing their autocleavage from the nascent polypeptide chain, before performing secondary cleavages leading to maturation of the viral replication machinery.[9–11]

3C^{pro} and 3CL^{pro} are chymotrypsin-like cysteine proteases, which have characteristic folding of two equivalent β-barrels to form the binding pocket and a catalytic triad or dyad.[12–14] Substrate recognition is typically dictated by a hexapeptide sequence (P_4–P_3–P_2–P_1–$P_{1'}$–$P_{2'}$), each amino acid interacting with its corresponding binding site (S_x) of the protease enzyme (Fig. 1A). The scissile bond is between the P_1–$P_{1'}$ residues which are typically glutamine (Gln)–glycine (Gly), or Gln–other small amino acid. Though cleavage occurs less commonly, glutamic acid (Glu)–glycine (Gly) is another peptide bond of interest.[15,16] Crystal structures of 3C^{pro} and 3CL^{pro} proteases along with substrate specificity studies have demonstrated subtle variation in the binding pockets and orientation of the catalytic triad/dyad, however all are remarkably conserved to recognise glutamine at P_1 position. Specificity for P_1 glutamine is attributed to conserved histidine (His) residue located at the end of the S_1 binding pocket which form important hydrogen bonds with the glutamine side chain of the natural substrate. Due to the critical roles that 3C and 3CL^{pro} play in viral replication and minimal homology with mammalian proteases, 3C^{pro} and 3CL^{pro} have emerged as promising targets for drug

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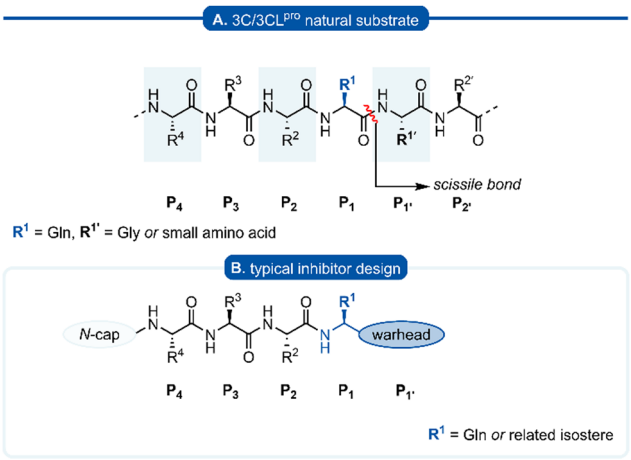


Fig. 1 (A) Hexapeptide recognition sequence of 3C/3CL^{pro}, where the scissile bond is located between P_1 (Gln) and $P_{1'}$. (B) Typical inhibitor design targeting 3C/3CL^{pro}.

development. A commonly employed approach in developing 3C/3CL^{pro} inhibitors is to mimic the natural substrate sequence, replacing the scissile bond with an electrophilic warhead to capture the catalytic cysteine residue, either *via* a reversible or irreversible mechanism. Peptidyl inhibitors targeting 3C/3CL^{pro} typically consist of a two to four peptide sequence (P_4–P_1), with glutamine occupying the P_1 position and an electrophilic "warhead" at $P_{1'}$ while the N-terminal is protected (N-cap) (Fig. 1B).

Following this blueprint, a number of low micromolar inhibitors targeting 3C/3CL^{pro} have been developed (representative examples, Fig. 2),[17–26] equipped with various electrophilic warheads demonstrating their effectiveness at trapping the catalytic cysteine residue. A drawback of employing a P_1 glutamine residue, however, is the high propensity for the amide side chain (Scheme 1A) to cyclise onto the electrophilic warhead, forming the hemiaminal tautomer (Scheme 1B). This reactivity has been observed with aldehyde,[17,27] α-haloketone,[23,28] and acyloxymethyl ketone[25] warheads containing P_1 glutamine or *N*-monoalkylated glutamine isosteres (see Section 1). Although the effects of tautomerisation to the hemiaminal are not clearly delineated, this is generally considered to be detrimental to antiviral activity while also causing complications during the chemical synthesis and purification. To avoid complications caused by the P_1 glutamine residue in the design of 3C/3CL^{pro} inhibitors, incorporation of a glutamine isostere which binds specifically in the S_1 pocket while also preventing cyclisation onto the electrophilic warhead is desirable. This review reports the advances made in the design of inhibitors targeting viral 3C/3CL^{pro} of human viruses in the *Pisoniviricetes* class and focusses on the reported development of P_1 glutamine isosteres.

Section 1. P_1 *N*-alkyl glutamine inhibitors

Early work in the development of P_1 glutamine isosteres focused on alkylation of the glutamine side chain amide to retard its nucleophilicity and prevent cyclisation onto the electrophilic warhead. Selected examples of inhibitors possessing a mono- or di-substituted amide side-chain at P_1 position and equipped with various warhead units are presented in Fig. 3. Malcolm *et al.* explored tetrapeptide inhibitors with *N*,*N*-dimethyl-Gln at the P_1 position.[29]

This was proposed to prevent cyclisation onto the aldehyde warhead while also maintaining hydrogen bonding between the glutamine δ-carbonyl oxygen and His_{160} of hepatitis A virus (HAV) 3C^{pro}.[12,29] Inhibitor **1a** was based on the preferred substrate sequence and was found to be a reversible, slow binding inhibitor of HAV 3C^{pro}, while also being 50-fold less active against human rhinovirus (HRV) 3C^{pro}. Investigation of a series of P_1 glutaminal inhibitors targeting HRV found *N*,*N*-dimethyl-Gln containing inhibitor **2b** was ~50-fold more active compared with the corresponding P_1 glutamine analogue **2a**.[27] The low potency of **2a** was postulated to be due to the propensity for cyclisation to the less active hemiaminal tautomer (see Scheme 1). A similar study was conducted investigating Michael acceptor tetrapeptides for anti-HRV activity. In this study however, unmodified glutamine inhibitor **3a** was found to have superior activity (EC_{50} = 0.54 μM) compared with both *N*-methyl-Gln **3b** and *N*,*N*-dimethyl-Gln **3c** containing analogues (EC_{50} = 5.6 μM and 4.0 μM, respectively).[19] The discrepancies in these studies likely arise from the propensity of the unprotected glutamine side chain to cyclise onto the aldehyde warhead, while Michael acceptors have not been observed to undergo cyclisation with the P_1 glutamine residue.[18] A related series of *N*-alkylated glutaminal inhibitors, cyclic tertiary amide exemplified by **4** were also shown to be effective in targeting norovirus (NV) 3CL^{pro}.[30]

A glutamine *N*-alkylation strategy has also been employed to generate inhibitors equipped with an α-fluoromethyl ketone (FMK) warhead and targeting HAV[31] and severe acute respiratory syndrome coronavirus (SARS-CoV)[23,28,32] Compound **1b** was found to inhibit HAV 3C^{pro} and displayed a second order rate constant of $k_{obs}/[I] = 3.3 \times 10^2$ M^{-1} s^{-1} [31] while *N*,*N*-dimethyl-Gln analogue **5a** was shown to be a low micromolar inhibitor of SARS-CoV replication.[28] The analogous inhibitor **5b** containing an unmodified Gln-FMK unit was found to be predominantly cyclised in solution (as observed by ^{1}H NMR) and 200-fold less active against SARS-CoV in a National Institute of Allergy and Infectious Diseases (NIAID) screen. An inhibitor containing an *N*-methyl-Gln-FMK unit **5c** was on the other hand reported as active according to a personal communication in a footnote; however partial cyclisation in solution was observed by ^{1}H NMR and a series containing this *N*-methyl-Gln-FMK unit does not appear to have been pursued further. A series of glutamine-trifluoromethyl ketone inhibitors, represented by inhibitor **6a** (K_i >1000 μM), were all found to be at least partially cyclised in solution (as determined by ^{19}F NMR in $CDCl_3$), while some existed solely in their cyclic form. These exhibited only moderate anti-SARS-CoV activity[23] while later work from the same laboratory showed *N*,*N*-dialkyl analogues **6b** and **6c** to have improved, albeit still low activity (K_i = 21.0 μM, 34.1 μM, respectively).[32]

α-Ketophthalahydrazide warheads with P_1 *N*,*N*-dimethyl-Gln isosteres, as illustrated by **7**, were investigated for anti-HAV

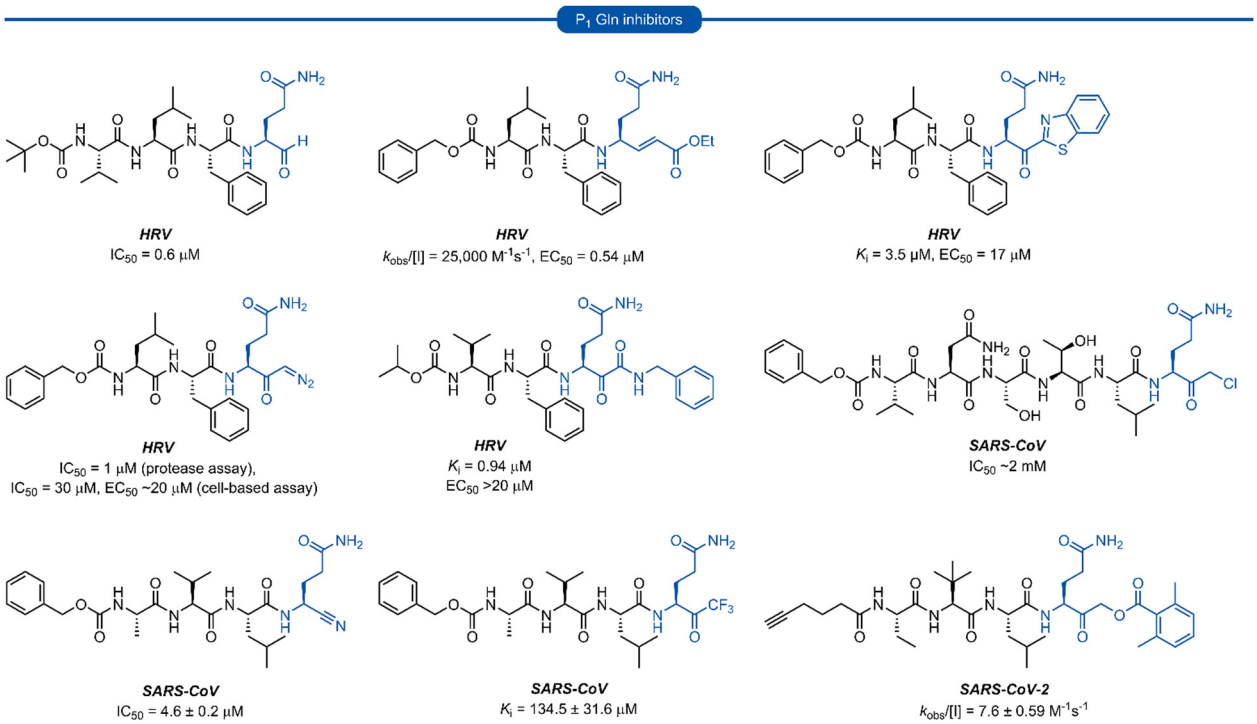
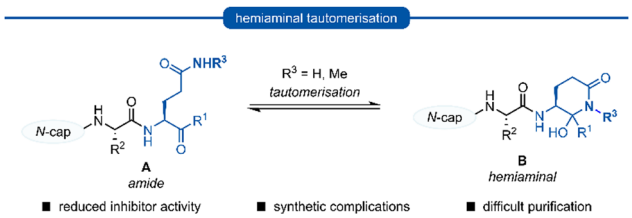


Fig. 2 Representative 3C/3CL^{pro} inhibitors possessing a P_1 (L)-glutamine residue with various electrophilic warheads.



Scheme 1 Generalised formation of hemiaminal B *via* tautomerisation of P_1 glutamine inhibitors A onto the electrophilic warhead.

3C^{pro} activity.[13,33] Intermolecular hydrogen bonding of the ketone and proximal hydrazide NH was postulated to restrict conformational mobility, thereby decreasing the entropic penalty upon binding, while also mimicking the $P_{2'}$ phenylalanine (Phe) of the natural substrate.[33] Compound **7** was found to be a competitive reversible inhibitor against HAV (IC_{50} = 13 μM), and no loss of activity was observed in the presence of a 100-fold excess of dithiothreitol (DTT), indicating its stability to extracellular thiols.

Section 2. P1 methionine derived inhibitors

Methionine (Met) and its oxidised sulfoxide and sulfone derivatives have also been investigated as non-nucleophilic glutamine P_1 isosteres due to their ease of synthesis and H-bonding ability (Fig. 4). Methionine sulfone analogue **8a** was found to be a good reversible inhibitor of HRV 3C^{pro} (K_i = 0.47 μM) and also displayed good antiviral activity in tissue culture assays (IC_{50} = 3.4 μM) with no observable cytotoxicity.[34] Additionally, inhibitor **8a** was found to be more potent than inhibitors **8b** and **8c** containing a P_1 methionine or glutamine residue, respectively (IC_{50} = 9.7 μM and IC_{50} = 42 μM).

Similar observations were made in a separate study investigating aldehyde HRV 3C^{pro} inhibitors. Sulfoxide **9c** displayed superior potency compared to analogue **9a** containing an unmodified Gln residue (IC_{50} = 0.005 μM *vs.* 3.6 μM), and was approximately equipotent to the *N*,*N*′-dimethyl-Gln analogue **9b**. The potency of sulfoxide **9c** was further supported by X-ray crystal structure data of the enzyme-inhibitor complex, which revealed the P_1 sulfoxide moiety fits snugly into the S_1 pocket and the H-bonding pattern of the active site His_{161} and Thr_{142} residues was analogous to that of the natural substrate.[27] The use of sulfoxide and sulfone P_1 isosteres in conjunction with Michael acceptor warheads on the other hand has met with less success. Two separate studies both reported that peptidyl inhibitors of HRV 3C^{pro} containing a P_1 sulfoxide **10a** or sulfone **10b** and **11b** residue showed a significant reduction in potency compared with their P_1 glutamine counterparts **3a** and **11a**, respectively.[19] Monopeptidyl aza-carboxamides, exemplified by **12a/b** (For aza-backbone modifications, see Section 5) have also been investigated for anti HRV and HAV activity. P_1 sulfone **12b** was found to have improved activity against HRV 3C^{pro} compared to *N*,*N*′-dimethyl-Gln analogue **12a**. Conversely, **12a** was found to exhibit slightly higher potency against HAV 3C^{pro} over **12b**.[35,36]

Section 3. P_1 inhibitors derived from non-proteogenic amino acids

A number of P_1 glutamine isosteres derived from unnatural amino acids were investigated during the early development of

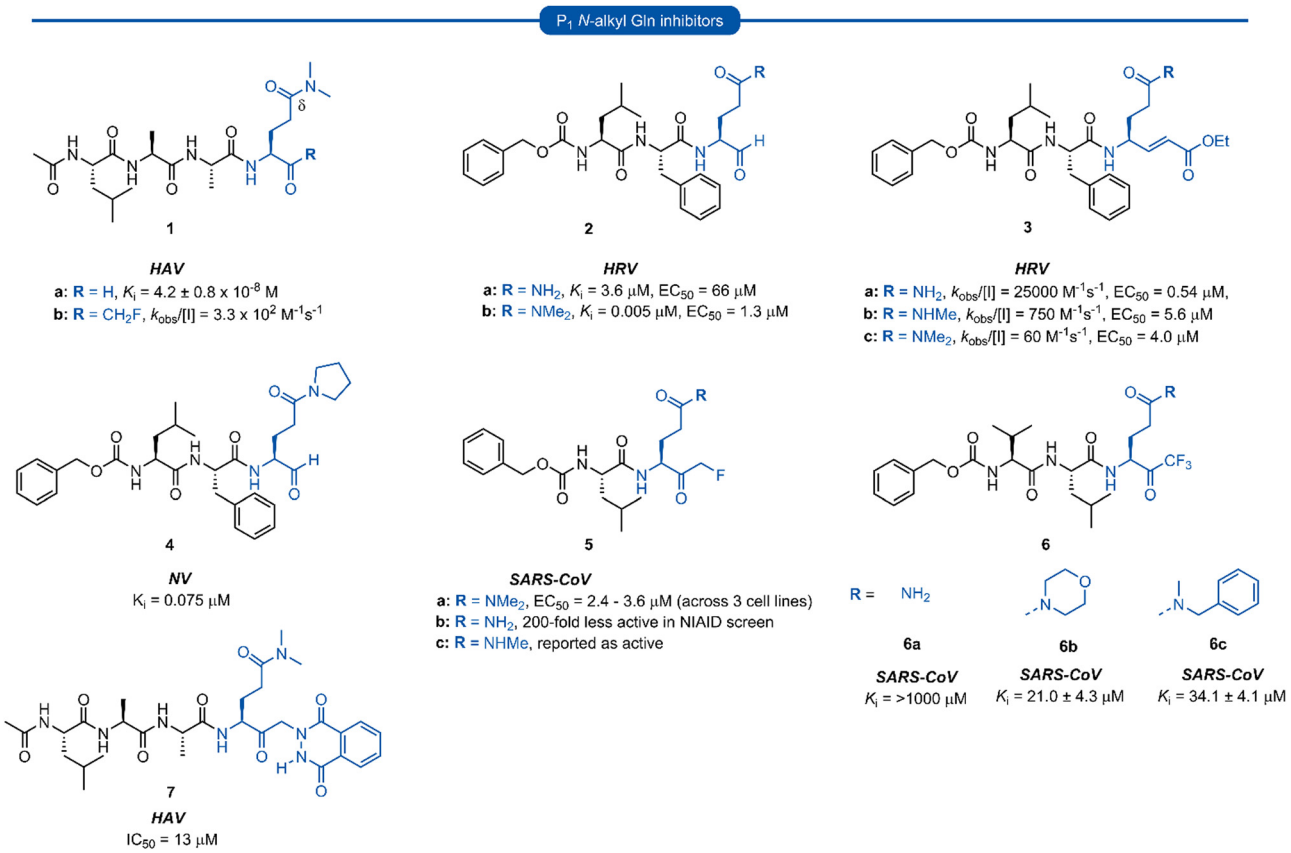


Fig. 3 Representative examples of P_1 *N*-alkyl-Gln inhibitors targeting 3C/3CL^{pro}.

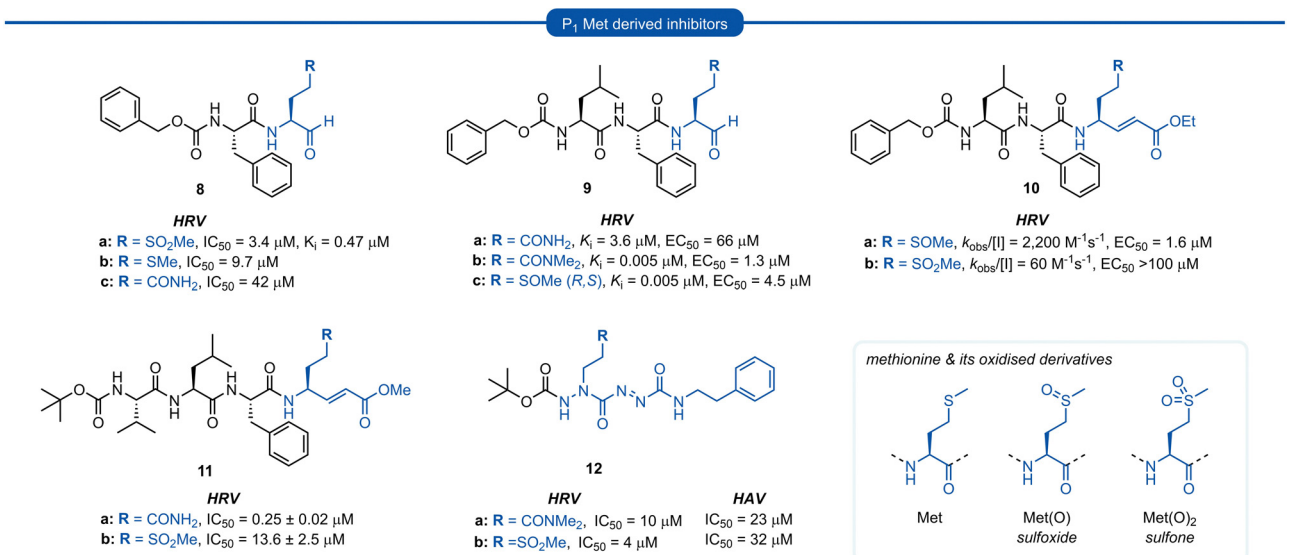


Fig. 4 Representative examples of P_1 Met derived inhibitors and their P_1 Gln/*N*-alkyl-Gln analogues targeting 3C/3CL^{pro}.

3C/3CL^{pro} inhibitors. Most notably, publications by Dragovich *et al.* and Webber *et al.* explored a diverse array of P_1 derivatives including glutamine related P_1 substrates (Section 1) and non-glutamine derived P_1 residues such as sulfones/sulfoxides (Section 2), as well as a number of glutamine isosteres (representative examples, Fig. 5A). During these studies however, none of these analogues **13–15** or **16–18** showed increased potency over inhibitors containing the unmodified side chain

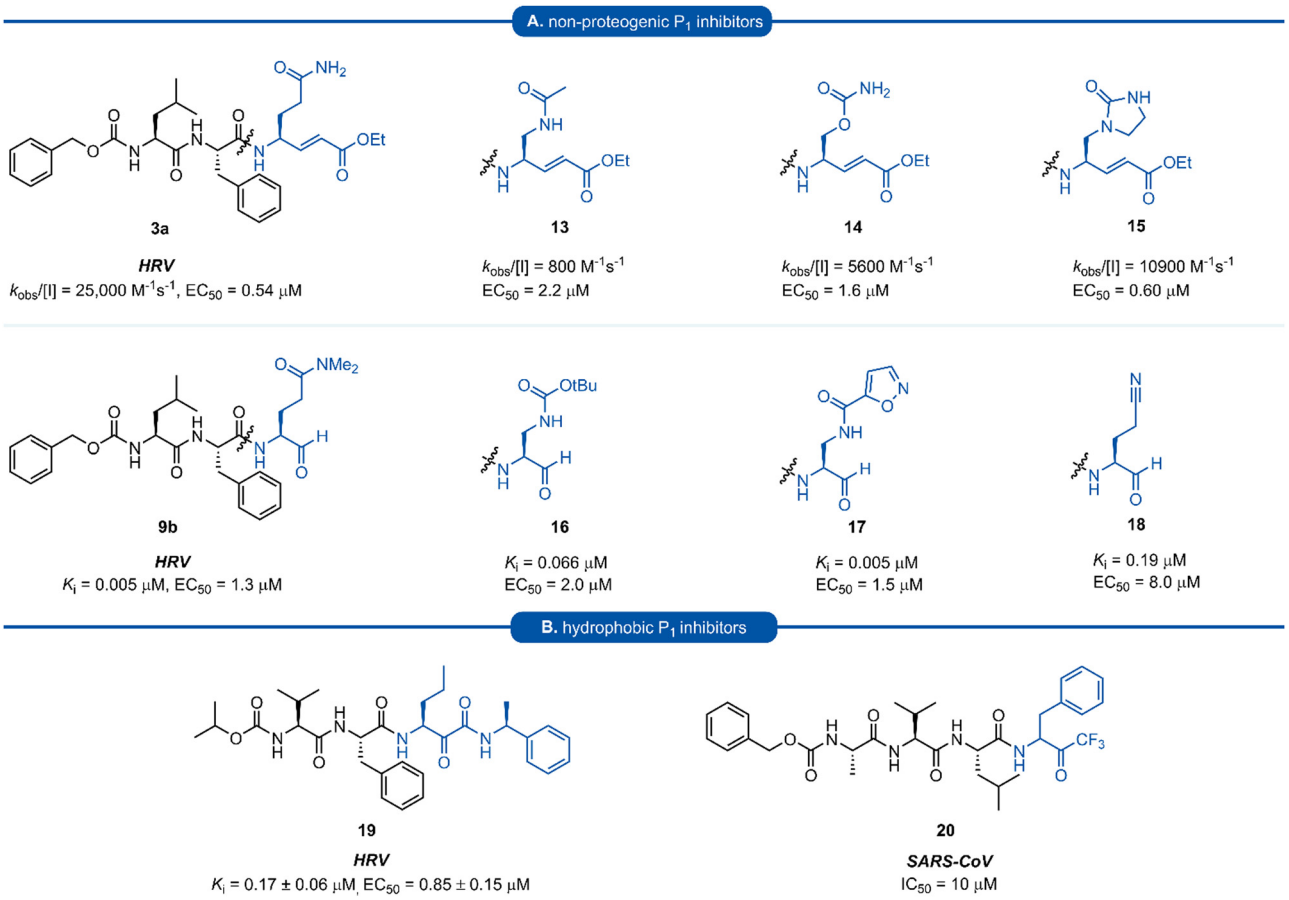


Fig. 5 (A) Inhibitors targeting 3C/3CL^{pro} containing non-proteogenic P_1 isosteres. (B) Hydrophobic P_1 residue inhibitors targeting 3C/3CL^{pro}.

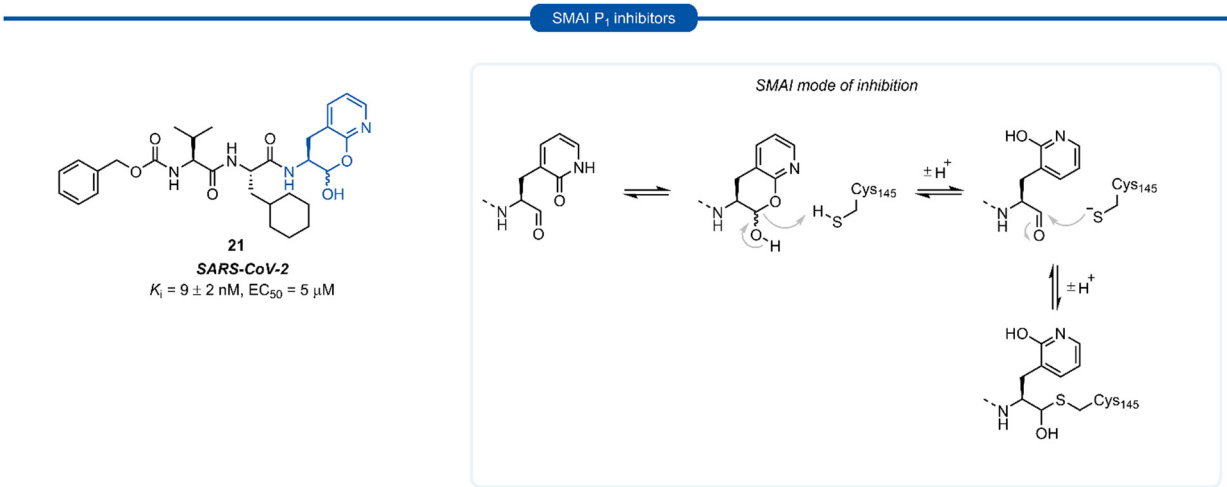
3a[19] or the *N*,*N*′-dimethyl-Gln **9b**[27] derivatives, respectively. Alternatively, peptidyl inhibitors bearing a hydrophobic amino acid in the P_1 position have also been investigated (Fig. 5B). The substitution of P_1 glutamine with non-nucleophilic residues circumvented synthetic complications caused by the reactivity of the electrophilic warhead. Representative examples **19** and **20** possess a norvaline (Nva) or phenylalanine hydrophobic amino acid at the P_1 position, respectively. Norvaline P_1 tetrapeptide inhibitor **19** was found to have low micromolar activity against HRV 3C^{pro} (K_i = 0.17) and also having good activity in tissue culture (EC_{50} = 0.85 μM),[24] while P_1-phenylalanine inhibitor **20** was found to exhibit moderate time-dependent activity against SARS-CoV 3CL^{pro} (IC_{50} = 10 μM).[37] A recent development in the inhibitor design has been made using self-masked aldehyde inhibitors (SMAIs) methodology for targeting 3CL protease.[38] Originally developed by Meek and co-workers to target cruzain, SMAIs was then applied to the development of SARS-CoV-2 inhibitor **21**.[39] SMAIs methodology is designed to mask the reactive aldehyde warhead through the spontaneous formation of a δ-lactol *via* nucleophilic attack by the P_1 2-pyridone side-chain on the aldehyde warhead, which would closely mimic the γ-lactam P_1 moiety that is now commonly employed in the design of 3C/3CL protease inhibitors (see Section 7). Upon binding to the protease active site, acid catalysed ring-opening by an acidic residue, revealing the reactive aldehyde warhead, which captures the cysteine residue to afford the enzyme-bound moiety (Scheme 2).

Formation of the lactol moiety was confirmed by NMR in organic solvents, however, unambiguous confirmation of the major species in aqueous solutions has not been made.[38] Compound **21** was found to be a competent inhibitor of SARS-CoV-2 with EC_{50} value of 5 μM. A high-resolution X-ray crystal structure of **21** bound in the active site of SARS-CoV-2 3CL^{pro} confirmed the formation of the anticipated hemithioacetal between the aldehyde group and Cys_{145}, thereby confirming the inhibitor's mechanism of action. The P_1 2-pyridone residue also establishes the expected H-bonding pattern, with the 2-pyridone carbonyl group accepting H-bonding from His_{163} and Ser_{144} while the amide proton donates an H-bond to Glu_{166} and Phe_{140}.[38]

Section 4. P_1 histidine inhibitors

During work towards the development of SARS-CoV 3CL^{pro} inhibitors, investigations into the substrate preference for SARS-CoV 3CL^{pro} *via* positional scanning of synthetic recombinant libraries based on the 7-amino-4-carbamoyl-coumarin (ACC) fluorogenic leaving group were undertaken.

A surprising preference for histidine at P_1 was revealed which was further confirmed by synthesising single substrates and comparing their kinetic constraints (Fig. 6).[40] Early investigations



Scheme 2 Putative mechanism of reversible covalent inhibition of a cysteine protease by SMAIs.

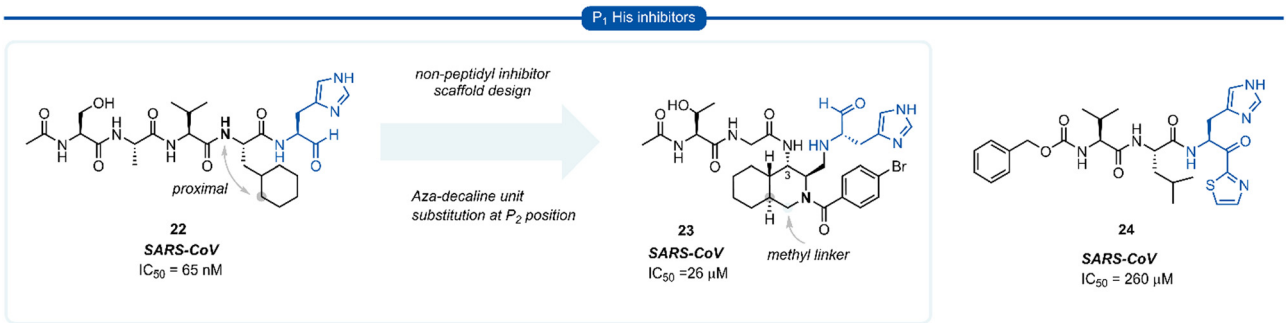


Fig. 6 Representative examples of P_1 His inhibitors targeting SARS-CoV $3CL^{pro}$.

of histidine P_1 analogues for anti SARS-CoV activity initially led to the discovery of the potent tetrapeptide inhibitor **22** (IC_{50} = 65 nM).[41] Analysis of the binding pocket of the inhibitor **22**-bound X-ray crystal structure showed that the cyclohexyl ring of the P_2 cyclohexylalanine (Cha) was proximal to the α-nitrogen of the corresponding amino acid residue. A novel decahydroisoquinoline inhibitor scaffold was designed by introduction of a methyl linker between the P_2 cyclohexyl ring and the P_2 backbone nitrogen and a peptide chain extending off the 3-position of the ring system of compound **23**. Although compound **23** represents a novel class of $3CL^{pro}$ inhibitors, improved potency against SARS-CoV $3CL^{pro}$ compared with its linear predecessor **22**, was not achieved.[42–44] Histidine P_1 residues were also employed by Hayashi *et al.* against $3CL^{pro}$ of SARS-CoV using α-keto-thiazole warheads **24**, however, these did not show superior activity to analogous P_1 lactam inhibitors (see Section 7) and were not further explored.[45]

Section 5. P_1 aza-glutamine inhibitors

Azapeptides are peptide chains where one of the amino acid residues are replaced by a semicarbazide which conformationally restricts the peptide, bending the amino acid away from the natural linear geometry. Incorporation of semicarbazides into peptidyl inhibitor manifolds has been shown to improve activity and selectivity while also showing improved pharmacokinetic properties such as metabolic stability and duration of action.[46] The success of aza-backbone modifications at the P_1 position of papain inhibitors initially led to their incorporation in the design of inhibitors for the closely related $3C/3CL^{pro}$ (Fig. 7).[47,48] Norbeck *et al.* developed P_1 aza-glutamine bromomethylketone **25** for anti-HRV activity as a time-dependent irreversible inhibitor (k_{inact}/K_{inact} = 23 400 M^{-1} s^{-1}).[49,50] In a separate study, a series of aza-glutamine inhibitors such as **26** were found to have moderate activity against both HRV and HAV $3C^{pro}$ (IC_{50} = 12 μM and IC_{50} = 10 μM) respectively.[35] Although a number of P_1 aza-glutamine inhibitors have been investigated, generally only moderate potency has been achieved against HRV and HAV.[48,51] P_1 aza-glutamine inhibitors have also been developed against SARS-CoV. Notably, James *et al.* demonstrated through X-ray crystal structure elucidation that aza-glutamine epoxide **27** irreversibly binds *via* an induced-fit model.[52,53] Müller and co-workers have also developed a potent inhibitor of $3CL^{pro}$ SARS CoV-2 containing a *N*-methyl-azanitrile warhead unit **28** (K_i = 24 nM).[54]

Section 6. P_1 Gln macrocyclic inhibitors

Macrocyclic inhibitors have been shown to display improved properties over their acyclic counterparts for the inhibition of

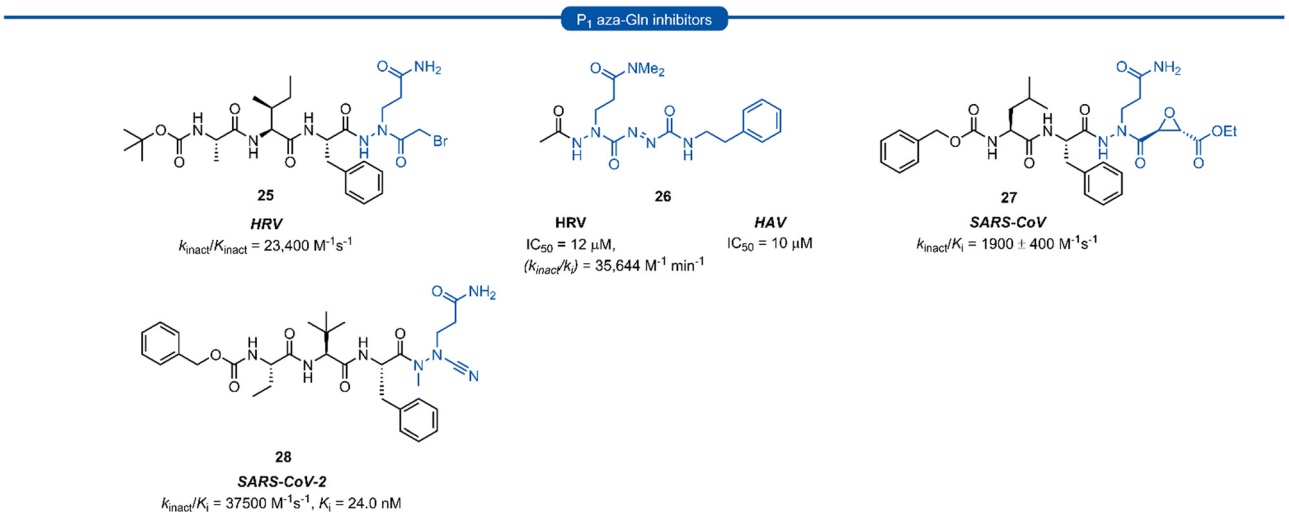


Fig. 7 Representative examples of P_1 aza-Gln inhibitors targeting 3C/3CL^{pro}.

protease enzymes. Protease enzymes recognise their ligands in a β-strand conformation, and macrocyclization can help facilitate the backbone hydrogen bonding pattern of peptide inhibitors to mimic that of an extended antiparallel β-sheet of the natural substrate.[55–57] Macrocyclization may also increase the structural rigidity of peptide-based inhibitors, restricting the conformational interchange and thereby enhancing the affinity for the receptor by reducing the loss of entropy upon binding, as well as exhibiting improved receptor selectivity and favourable drug-like characteristics including increased cell permeability and proteolytic stability.[55,58–60] The first investigations into macrocyclic 3C^{pro} and 3CL^{pro} inhibitors arose from analysis of the crystal structure of inhibitors bound to HRV 3C^{pro}, revealing close spatial proximity between P_1 and P_3 side-chains. Modelling studies yielded tripeptidyl macrocyclic inhibitor **29**, where a triazole-containing linker adjoined the P_1 and P_3 residues (Fig. 8). The inhibitor activity was evaluated against norovirus, enterovirus and SARS-CoV, and in all cases **29** was found to inhibit the protease in the low micromolar range.[61] Further investigation showed poor correlation between 3CL^{pro} enzyme inhibition assays (IC_{50}) and inhibition of norovirus replication in a cell-based replicon system assays (EC_{50}) for **29**

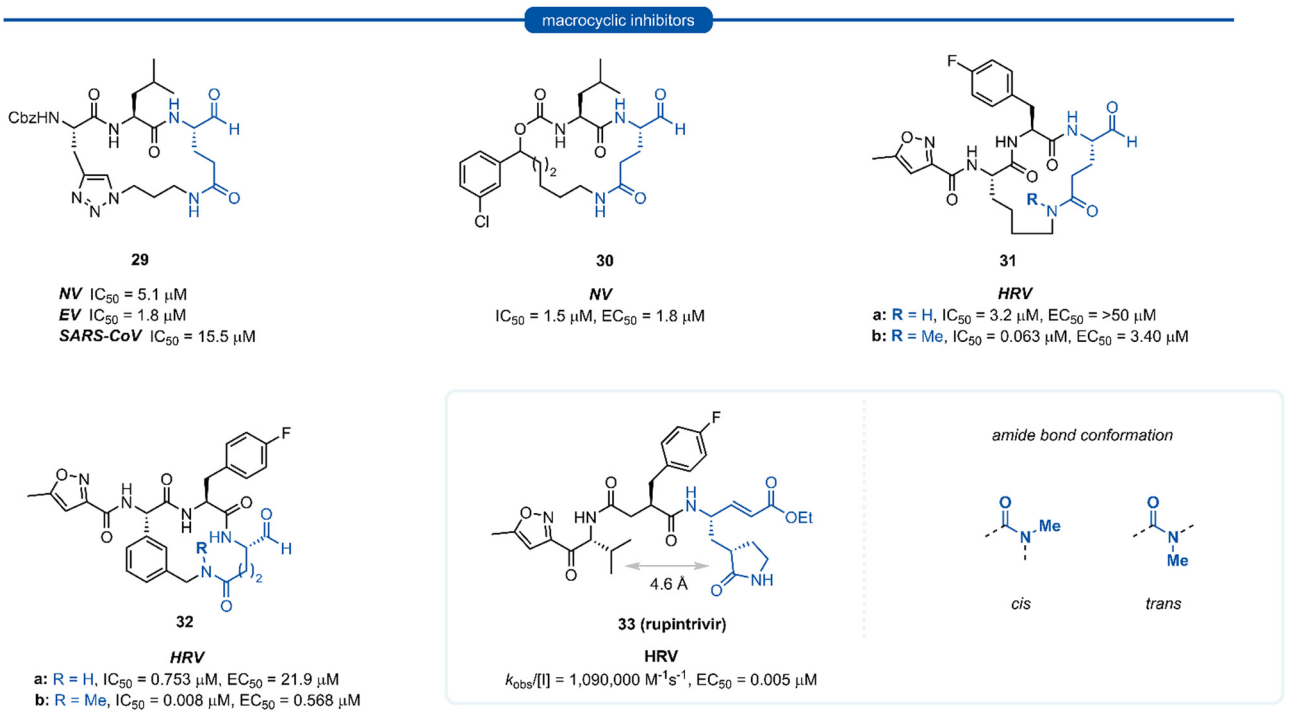


Fig. 8 Representative examples of macrocyclic lactam inhibitors containing a P_1 Gln residue.

and derivatives thereof.[62] This result indicates that compounds such as **29** likely suffer from poor cellular permeability due to the triazole ring increasing the polar surface area. An X-ray crystal structure of NV 3CL^{pro} with bound inhibitor **29** revealed the expected antiparallel β-sheet backbone binding conformation, and the tetrahedral adduct between the aldehyde warhead and Cys_{139} residue was also present. It was noted however, that binding induced suboptimal structural changes of the protease binding pocket and more importantly, the S_1 pocket, resulting in loss of the key Thr_{134} and His_{157} H-bonds to P_1 Gln and thus maximal binding efficiency was not obtained.[62,63] Further publications explored related macrocyclic inhibitors with alkyl linkers which generally gave increased cell permeability, with inhibitor **30** found to have good activity in both enzyme and cell-based assays. Co-crystallisation of **30** and NV 3C^{pro} showed the expected backbone H-bonding pattern indicating the expected bonding orientation. The key H-bonding interaction between the P_1 amide of **30** and His_{157} and Thr_{134} of NV 3C^{pro} however was again absent; instead, a new H-bond between the oxygen of the tetrahedral thioacetal adduct and Pro_{136} was evident.[64] Rupintrivir **33** (AG-7088), a potent anti-rhinoviral agent that became the subject of extensive preclinical and clinical investigation, was used as a scaffold for macrocyclic inhibitors, with the goal of improving rupintrivir's pharmacokinetic properties.[65] The crystal structure of rupintrivir/HRV2 3C^{pro} showed only 4.6 Å between the P_1 and P_3 side chains, providing an opportunity for the introduction of a macrocyclic linkage.

The resulting macrocycle **31a** was tested against HRV 3C^{pro} and showed decreased potency compared to rupintrivir ($EC_{50} > 50$ μM) in HeLa cells infection model, albeit conserving moderate protease inhibition activity with IC_{50} values in the low micromolar range (IC_{50} = 3.2 μM). Modelling of the macrocyclic peptide in the binding pocket predicted the glutamine-derived amide bond was required to be in the *cis* conformation for efficient binding. Despite the *cis* conformation being higher energy, methylation of the amide nitrogen allows increased population of the *cis* conformer. Inhibitor **31b** containing an *N*-methyl moiety showed a 50-fold increase in potency against HRV 3C^{pro} (IC_{50} = 0.063 μM) despite losing an H-bonding interaction between the P_1 amide proton and backbone carbonyl of Thr_{142}. Alternatively, incorporation of a phenyl ring into the macrocyclic linkage gave inhibitor **32a** (IC_{50} = 0.753 μM), which displayed a 4-fold increase in activity over **31a**. Additional incorporation of the *N*-methyl moiety afforded **32b** and further increased the activity by 100-fold (IC_{50} = 0.008 μM).

Section 7. P_1 lactam inhibitors

Pioneering work by Dragovich and co-workers first led to the use of γ-lactams as P_1 glutamine isosteres in a series of publications developing tripeptide inhibitors of HRV 3C^{pro}.[18,19,66,67] Early investigations (see Sections 3 and 4) found that none of the P_1 non-glutamine inhibitors showed improved potency over the inhibitor containing an unmodified glutamine side chain **3a** (Fig. 9A). Inspection of the X-ray crystal structure of **3a** bound to HRV 3C^{pro} revealed that while the P_1 *trans* amide proton is engaged in a H-bond to the backbone carbonyl of Thr_{142}, the *cis* amide proton is solvent exposed, providing a vector for further optimisation of the P_1 side chain. *N*-Monomethyl inhibitor **3b** displayed significantly weaker protease inhibition, presumably due to the amide preferentially adopting a *trans* conformation, interrupting the key P_1 amide proton-Thr_{142} H-bond (Fig. 9A). In order to lock in the desired *cis* amide conformation, an (*S*)-γ-lactam was incorporated into the P_1 side chain.

The resulting tripeptide **34a** displayed a remarkable increase in protease inhibition activity against HRV 3C^{pro} as well as submicromolar antiviral activity in cell culture.[67] The combination of this newly optimised P_1 side chain with favourable P_2–P_4 modifications discovered in related work gave rise to rupintrivir **33** (AG-7088). During the initial investigation into P_1 lactam-based glutamine isosteres, it was found that while a (*S*)-δ-lactam moiety **34b** was equally effective as the (*S*)-γ-lactam **34a**, switching the stereochemistry of the lactam ring **34c** or replacing the lactam moiety with a 5-membered cyclic urea **34d** resulted in a significant reduction in activity (Fig. 9B).[67] Following the work of Dragovich *et al.* and guided by crystal structure studies revealing the high degree of structural homology among 3C^{pro} and 3CL^{pro} enzymes, P_1 (*S*)-lactam isosteres have been integrated into the design of numerous inhibitors targeting 3C/3CL^{pro}. The first example outside of the preliminary work on HRV protease was for the development of inhibitors of hepatitis A virus (HAV) 3C^{pro}. From a small series of compounds bearing an α-ketophthalahydrazide, compound **35** proved to be the most effective (IC_{50} = 1.6 μM).[68] Representative examples of 3C/3CL^{pro} inhibitors bearing a P_1 (*S*)-lactam isostere are shown in Fig. 10, showcasing the compatibility of the P_1 (*S*)-lactam with a range of electrophilic warhead functionalities. The (*S*)-lactam P_1 unit has been shown to be effective in targeting numerous viral 3C/3CL^{pro} including compounds **36–38** for enterovirus,[69–74] **39** for norovirus,[75–79] **40–41** for SARS-CoV,[32,45,80–89] **42** for MERS-CoV,[90–92] and **43–51** for SARS-CoV-2 (Fig. 11). A handful of inhibitors demonstrating broad spectrum activity have also been developed.[93–96] As represented in Fig. 10, 3C/3CL^{pro} inhibitors typically employ a (*S*)-γ-lactam P_1 isostere, however enterovirus has been shown to prefer the (*S*)-δ-lactam isostere at the P_1 position. This was first demonstrated through docking studies of EV71 3C^{pro}, revealing the δ-lactam had shorter H-bond distances with His_{161} and Thr_{142} compared to the γ-lactam, while also adopting a preferable binding orientation in the S_1 pocket.[70,71] This model was validated through the design of inhibitor **36c**, which was 7–10 fold more active than the corresponding γ-lactam **36b**. In a separate study, screening of a compound library revealed compound **37** containing a chiral cyanohydrin warhead.[72] Each epimer was tested separately and exhibited good activity against EV71 with little effect of the warhead stereochemistry on the potency of the inhibitor. The same library screening also revealed an inhibitor scaffold analogous to that of **37** with an aldehyde warhead **38**, to be the most potent compound tested against EV71 in both 3C^{pro} protease inhibition assay and human rhabdomyosarcoma cells viral infection model.[70–72]

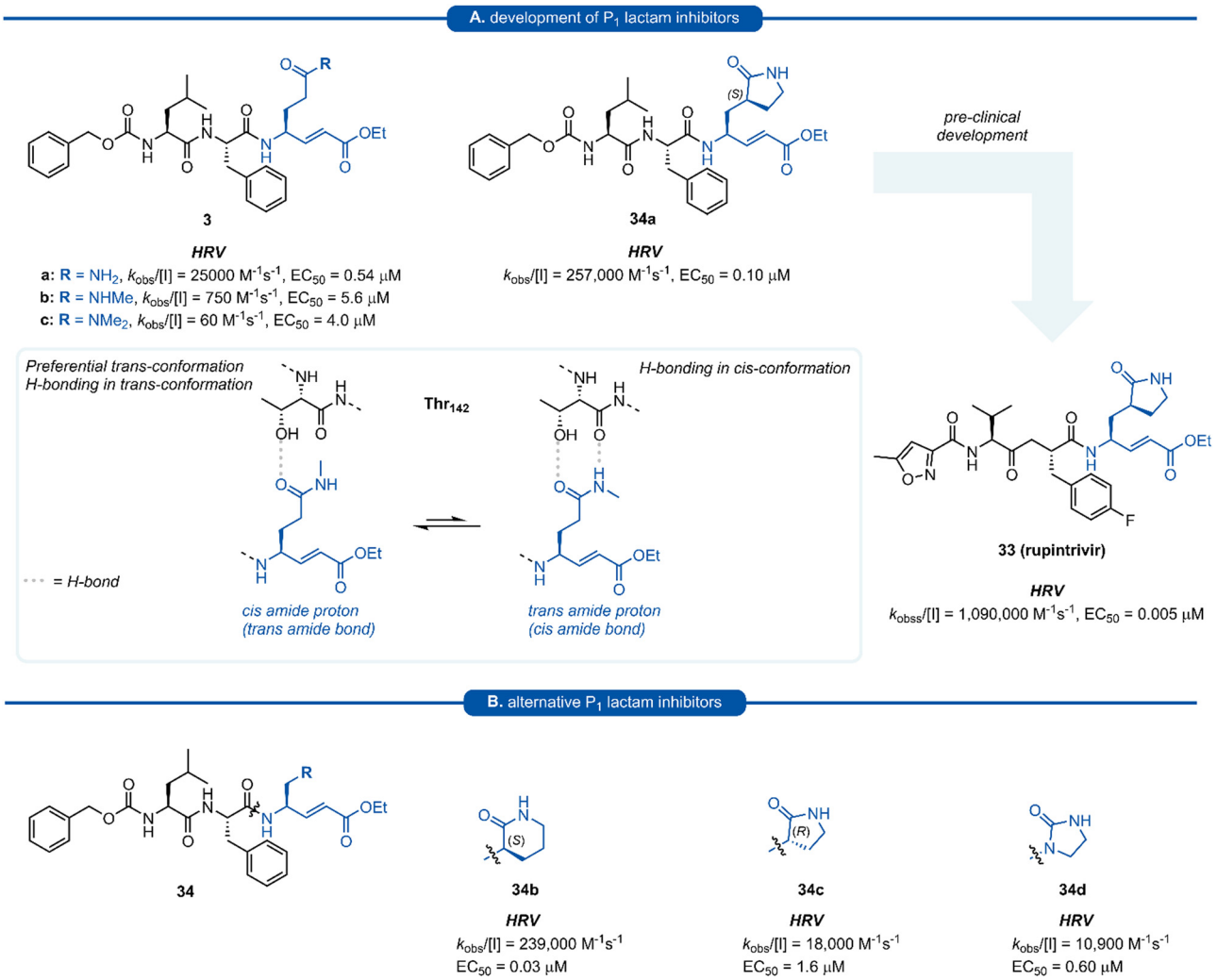


Fig. 9 (A) Development of HRV inhibitors possessing a P_1 (*S*)-γ-lactam moiety. (B) Alternative P_1 lactam isosteres.

Since the SARS-CoV-2 outbreak in 2019, new inhibitors of the main protease $3CL^{pro}$ (aka M^{pro}, Nsp5) have almost exclusively been developed using P_1 (*S*)-γ-lactam isostere. In early work, it was realised that a high degree of homology existed between $3CL^{pro}$ of SARS-CoV and SARS-CoV-2.[97] In addition to their 96% sequence identity, X-ray crystal data has demonstrated there to be a high structural similarity between SARS-CoV and SARS-CoV-2 $3CL^{pro}$ with a root mean square deviation of 0.53 Å across all Cα positions.[98] This suggested that structural features from known coronaviral protease inhibitors are likely to be effective when incorporated into the design of SARS-CoV-2 $3CL^{pro}$ inhibitors. This was demonstrated in an early report by Hilgenfeld and co-workers in the development of α-ketoamide inhibitor **43** from known inhibitor **42**. The (*S*)-γ-lactam moiety was found to occupy the $3CL^{pro}$ S_1 pocket, with the crystal structure of SARS-CoV-2 $3CL^{pro}$ with **43** highlighting the crucial H-bonding between the lactam carbonyl oxygen with His_{163} side-chain imidazole, and the lactam nitrogen forming a three-centred hydrogen bond with Phe_{140} and Glu_{166}.[98] Various electrophilic warhead moieties have been demonstrated as effective in combination with a P_1 (*S*)-γ-lactam group targeting SARS-CoV-2 $3CL^{pro}$. Dipeptides bearing a heterocyclic acyloxymethylketone warhead were shown to be excellent irreversible inhibitors of SARS-CoV-2 $3CL^{pro}$, exemplified by **44**.[99] Compound **44** also exhibited excellent plasma stability, glutathione stability, and selectivity over human proteases cathepsin B and cathepsin S. Inhibitor **45**, which possesses a Michael acceptor warhead unit and inhibits SARS-CoV-2 $3CL^{pro}$ in the low micromolar range (IC_{50} = 0.9 μM), was recently demonstrated to exhibit significantly superior antiviral activity in hACE2 cell assays (EC_{50} = 8.2 nM).[100] Upon further investigation, this enhanced potency was attributed to a dual mode of action of compound **45** in targeting the SARS-CoV-2 virus lifecycle. In addition to inhibiting $3CL^{pro}$, **45** was found to also inhibit cathepsin L (CatL), a protease enzyme responsible for cleaving the viral Spike protein, promoting cell entry. Intraperitoneal administration of **45** in SARS-CoV-2 infected transgenic mice also showed to cause reduced viral load in the lungs and enhanced survival rate. In line with the development of inhibitors of other viral proteases, the introduction of an

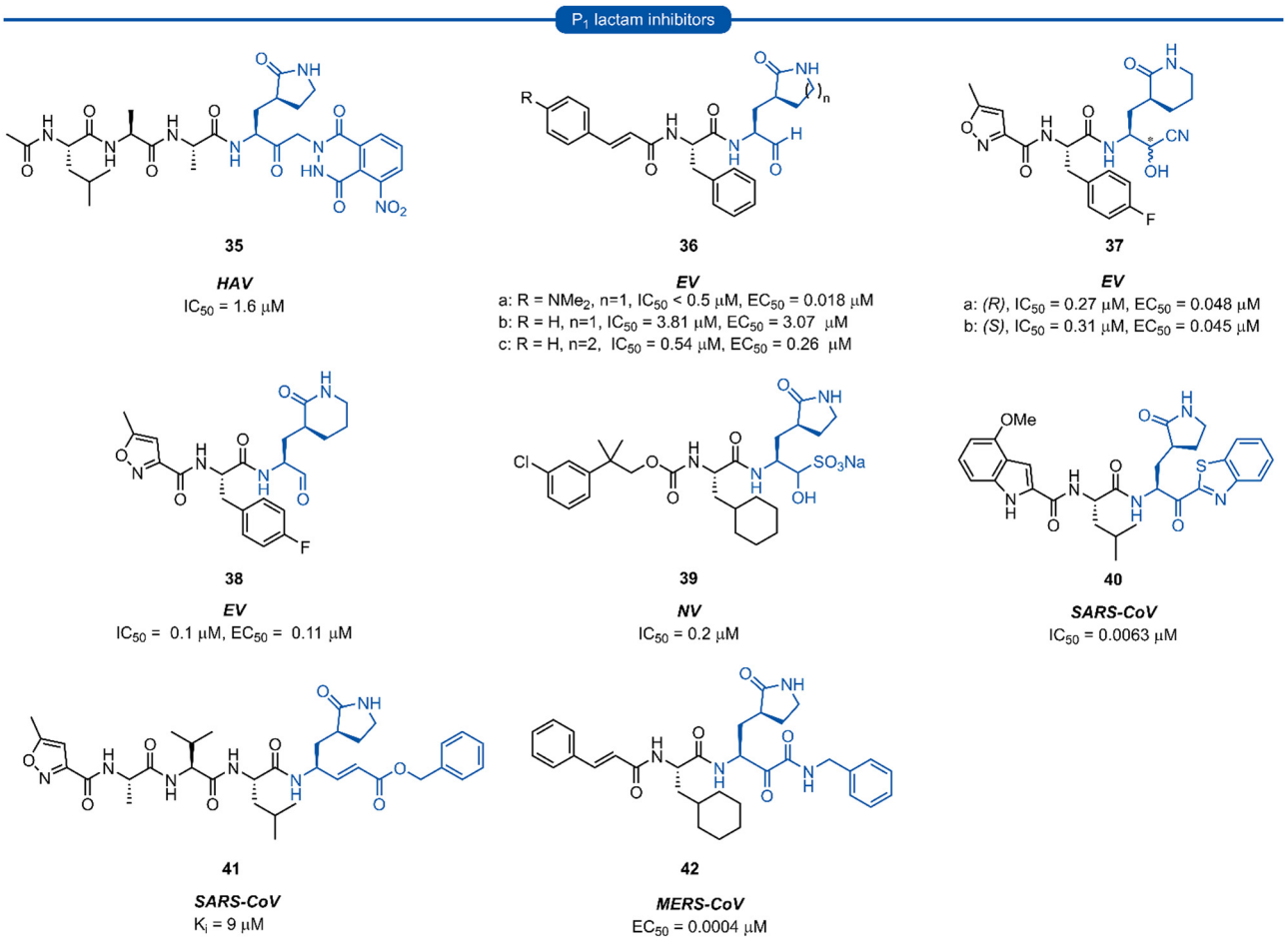


Fig. 10 Representative examples of inhibitors targeting 3C/3CL^{pro} containing a P_1 (*S*)-γ/δ-lactam isostere.

aldehyde C-terminal warhead has also proved effective for the discovery of SARS-CoV-2 3CL^{pro} inhibitors (**46–48**). Structure-based design utilising the crystal structure of SARS-CoV 3CL^{pro} gave rise to dipeptide aldehyde **46**, which demonstrates good anti-SARS-CoV-2 activity (EC_{50} = 0.53 nM) and was selected for further preclinical development.[101] Compounds **47** (*aka* MI-30)[102] and **48** (*aka* UAWJ9-36-3)[103] represent a series of aldehyde-based hybrid inhibitors which combine the P_1 (*S*)-γ-lactam group with a bicycloproline P_2 residue derived from the hepatitis C virus (HCV) inhibitors, telaprevir[104] (P_2 5,5-fused ring) or boceprevir[105] (P_2 3,5-fused ring), respectively. Unlike work towards other viral 3C/3CL^{pro} inhibitors highlighted in this review, SARS-CoV-2 inhibitors are now at various stages of clinical development (Fig. 11). Compound **49** (aka PF-00835231), containing a P_1 (*S*)-γ-lactam group in conjunction with a hydroxymethylketone C-terminal electrophilic warhead, was identified as a potent inhibitor of SARS-CoV-2 3CL^{pro} (IC_{50} = 4 nM) and was selected as the basis for the development of a protease inhibitor for the treatment of COVID-19.[106] One drawback of compound **49** that became apparent during preclinical characterisation was its relatively poor intrinsic aqueous solubility (<0.1 mg mL^{-1}), which was insufficient to formulate for intravenous (IV) infusion. Accordingly, IV administration was enabled by utilizing a prodrug strategy, resulting in the phosphate prodrug lufotrelvir (compound **50**) which displays vastly superior aqueous solubility of >200 mg mL^{-1} over a pH range suitable for IV infusion.[107] Lufotrelvir (**50**) is rapidly converted to the active free hydroxymethyl ketone (PF-00835231, **44**) following administration. Work towards development of an orally bioavailable SARS-CoV-2 inhibitor has been undertaken by Owen and co-workers (Pfizer). Again using **49** as a lead compound, several modifications were made in order to reduce the hydrogen bond donor (HBD) count which has been shown to correlate with poor oral bioavailability.[108] Changing the electrophilic warhead from a hydroxymethyl ketone (HMK) to a nitrile group significantly increased its oral bioavailability (Oral F = 1.4% *vs.* 7.6% for otherwise identical HMK and nitrile inhibitors, respectively). It was also noted that compounds containing a nitrile warhead were less likely to undergo epimerization at the P_1 group during chemical synthesis compared to those equipped with heterocyclic ketone warheads that were also being investigated.[108] Incorporation of a P_2 bicycloproline derivative and *N*-terminal cap modifications afforded nirmatrelvir (**51**), which displays superior activity (EC_{50} = 74.5 nM) and oral bioavailability (*F* = 50%) over its predecessor **49** (EC_{50} = 231 nM, oral *F* = 1.4%).[109]

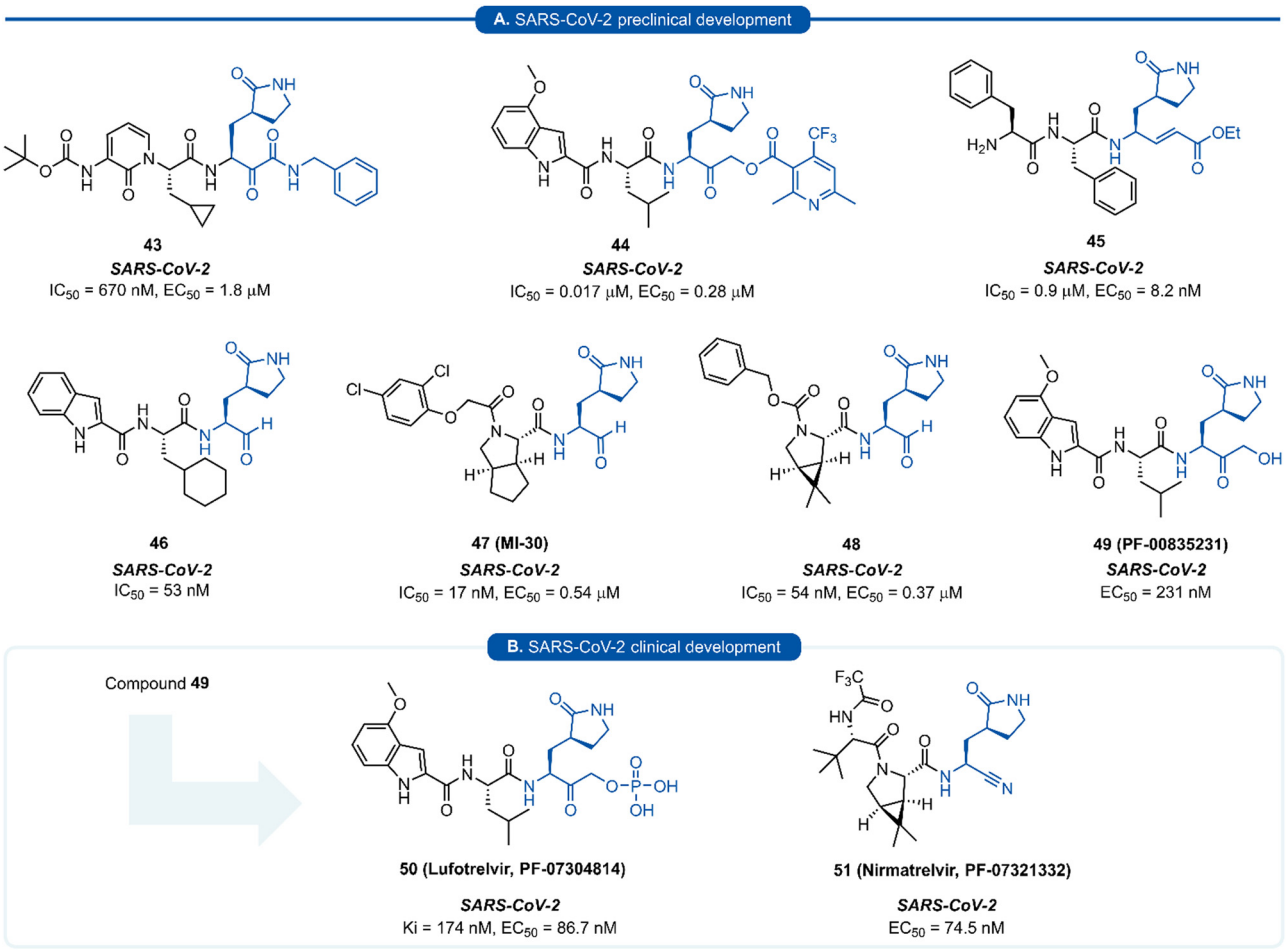
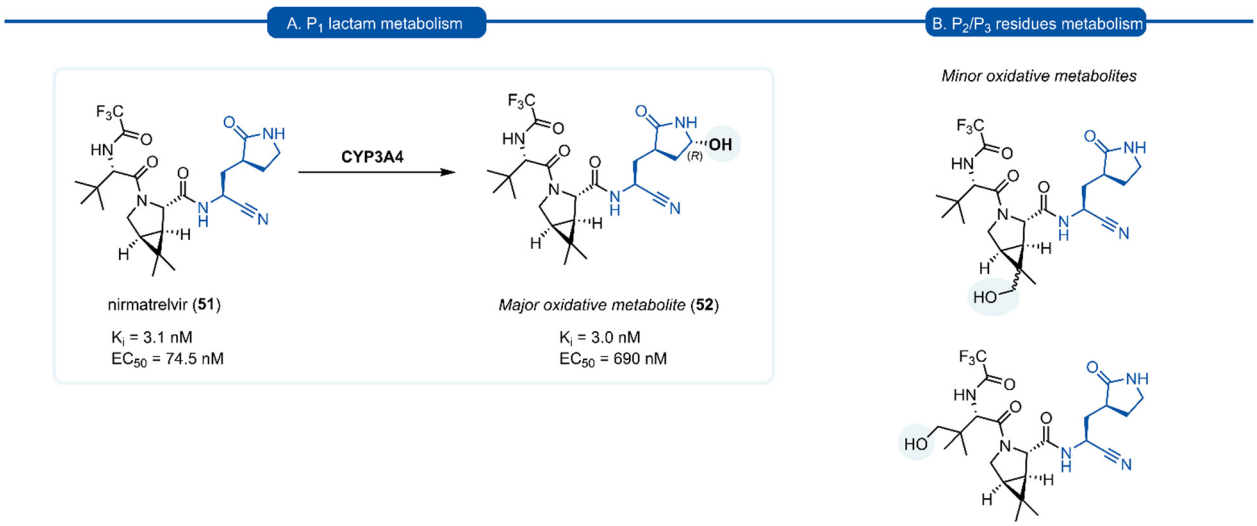


Fig. 11 Representative examples of SARS-CoV-2 inhibitors in preclinical (A) or clinical (B) development.

A drawback of the P_1 (*S*)-γ-lactam glutamine isostere which became apparent through preclinical development of nirmatrelvir (**51**), is its susceptibility to undergo cytochrome P450 (CYP450) oxidative metabolism. The primary route of inactivation of nirmatrelvir in human liver microsomes occurs *via* CYP3A4-mediated oxidation. The major metabolite **52** resulting



Scheme 3 (A) The primary route of metabolism of nirmatrelvir **51** *via* CYP3A4 hydroxylation of the P_1 (*S*)-γ-lactam moiety. (B) Minor metabolites products of nirmatrelvir **51** *via* CYP3A4 hydroxylation of the P_2 and P_3 moiety.

from hydroxylation of the pyrrolidine ring and which, albeit exhibiting similar inhibition potency against $3CL^{pro}$ and repressing viral replication, was 10-fold less active against SARS-CoV-2 compared to **51** in epithelial Vero E6 cells infection model (Scheme 3). In the same study, other minor metabolites of nirmatrelvir, produced by CYP3A4 *via* alternative oxidation patterns at the P_1 and P_2 position, were detected in trace amounts, but their individual inhibitory potencies were not measured due to their low relative abundance.[109] This has ultimately led to nirmatrelvir being co-administered with the CYP3A4 inhibitor, ritonavir, to increase its bioavailability. This combined formulation, Paxlovid™ has proven to be a safe and effective treatment for COVID-19 for patients with high risk of disease progression, resulting in an 89% risk reduction in treated patients relative to a placebo group.[110]

Conclusion

Over the last 25 years, considerable effort has been invested into the development of P_1 glutamine isosteres for incorporation into inhibitors targeting $3C^{pro}$ and $3CL^{pro}$ of viruses in the *Pisoniviricetes* class. Early efforts exploring P_1 *N*-alkyl glutamine inhibitors were met with mixed success, while more structurally diverse methionine and carbonyl derived isosteres of glutamine, were generally found to be detrimental to inhibitor activity. Macrocyclization and aza group backbone modifications at the P_1 position, although delivering low micromolar inhibitors, also did not demonstrate any significant improvements over their linear and unmodified counterparts. Incorporation of the P_1 lactam isostere resulted in significantly more potent inhibitors and the development of the (*S*)-γ-lactam moiety marked the first real progress towards potent $3C/3CL^{pro}$ inhibitors. The lactam was found to conformationally restrict the P_1 side chain amide bond in the *cis* conformation, providing optimal H-bonding interactions in the S_1 binding pocket and reducing the entropic cost of binding. Moreover, the inhibitor inactivation by intramolecular cyclisation of the nucleophilic P_1 amide moiety onto the electrophilic warhead, was not observed for the (*S*)-lactam. The P_1 (*S*)-lactam moiety has now found widespread use in targeting $3C/3CL^{pro}$ due to its optimal binding interactions and broad compatibility with electrophilic warheads. Recent metabolic studies on nirmatrelvir, however, have now revealed the lactam group to be the primary metabolic hotspot. Although the P_1 lactam moiety has provided one of the major milestones in targeting $3C^{pro}$ and $3CL^{pro}$, further structural optimisation of the lactam side chain is desirable to improve the pharmacokinetics of P_1 lactam containing inhibitors.

Author contributions

L. A. Stubbing: concept and original version. J. Bell-Tyrer: writing – original draft. Y. O. Hermant, J. G. Hubert, S. H. Yang, A. M. McSweeney, G. M. McKenzie-Goldsmith, D. P. Furkert, V. K. Ward, M. A. Brimble: writing – review and editing. V. K. Ward, M. A. Brimble: project administration, funding acquisition.

Conflicts of interest

There are no conflicts to declare.

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