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Considerations for the discovery and development of 3-chymotrypsin-like cysteine protease inhibitors targeting SARS-CoV-2 infection

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Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the cause of the COVID-19 pandemic. The coronavirus 3-chymotrypsin-like protease (3CLpro) controls virus replication and is therefore considered a major target and promising opportunity for rational-based antiviral discovery with direct acting agents. Here we review first-generation SARS-CoV-2 3CLpro inhibitors PF-07304814, GC-376, and CDI-45205 that are being delivered either by injection or inhalation due to their low intrinsic oral bioavailability. In addition, PF-07321332 is now emerging as a promising second-generation clinical candidate for oral delivery. A key challenge to the development of novel 3CLpro inhibitors is the poor understanding of the predictive value of *in vitro* potency toward clinical efficacy, an issue complicated by the involvement of host proteases in virus entry. Further preclinical and clinical validation will be key to establishing 3CLpro inhibitors as a *bona fide* class for future SARS-CoV-2 therapeutics for both hospitalized and outpatient populations.

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Introduction

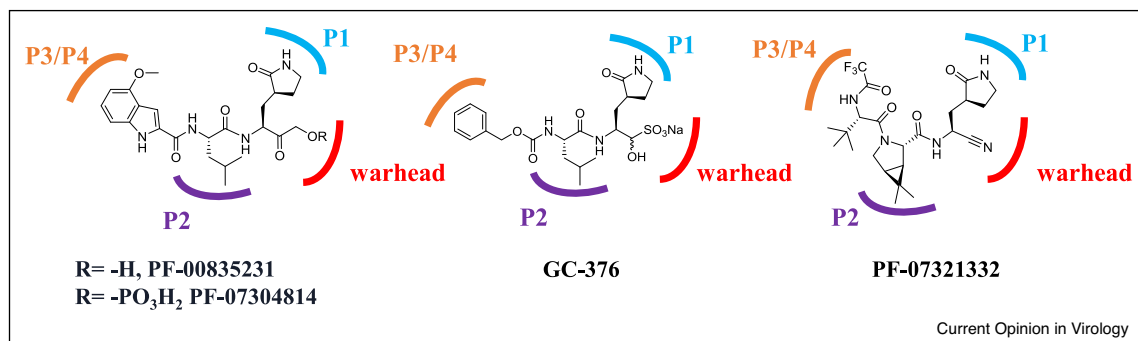
COVID-19 is a respiratory infection caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Although most infected individuals are either asymptomatic or experience self-limiting symptoms similar to the common cold, clinical cases of severe COVID-19 require hospitalization and intensive care due to pneumonia and extra-pulmonary manifestations [1,2]. The anti-inflammatory corticosteroid dexamethasone, and nucleotide analog remdesivir are

considered the standards of care for the treatment of severe COVID-19 in hospitalized patients needing supplemental oxygen (<https://www.covid19treatmentguidelines.nih.gov/therapeutic-management/>). However, these molecules are not suitable for patients in the early stage of SARS-CoV-2 infection, in outpatient settings, or as prophylaxis. SARS-CoV-2 is an enveloped, positive-sense, single-stranded RNA virus that belongs to the β -coronavirus genus of the *Coronaviridae* family [3]. The main viral protease, 3CLpro, is a cysteine protease with distinctive substrate preference for glutamine at the P1 site (Leu-Gln/(Ser,Ala,Gly)) [4]. Because of its key role in viral replication, 3CLpro is considered a major target for antiviral drug discovery. Intense research and development are currently ongoing to evaluate 3CLpro inhibitors as potential treatments for COVID-19.

Origin and status of first-generation 3CLpro inhibitors

The urgent need for COVID-19 treatments triggered rapid repurposing of protease inhibitors previously developed for other viral indications. FDA-approved HIV protease inhibitors do not provide any benefit for COVID-19 patients [5]. The most interesting subgroup of protease inhibitors are peptidomimetics previously designed to inhibit 3C-like or 3CL-like proteases. These molecules contain a P1-glutamine surrogate, like those designed for the discovery of the rhinovirus 3Cpro inhibitor rupintrivir [6]. While rupintrivir itself did not display significant activity against SARS-CoV-2 3CLpro [7], peptidomimetics previously described for other coronaviruses were expected to have broad activity across most coronaviruses as their substrate cleavage sites are highly conserved (for review: Ref. [8]). Unsurprisingly, some peptidomimetic compounds active against MERS and SARS-CoV-1 are also active against SARS-CoV-2. PF-00835231 was developed more than 15 years ago against SARS-CoV-1 (patent WO2005113580) and recently showed high *in vitro* potency against SARS-CoV-2 (Figure 1) [9,10**]. Currently its phosphate prodrug PF-07304814 is being evaluated in a Phase 1b clinical trial in hospitalized patients with COVID-19; safety and efficacy results are expected around mid-2021 (ClinicalTrials.gov Identifier NCT04535167). The peptidomimetic compound GC-376 is a broad-spectrum inhibitor of 3C and 3C-like proteases of picornaviruses, noroviruses and coronaviruses (Figure 1) [11*]. The compound has been licensed to Anivive, a company focusing on Companion animal veterinary medicine, to treat feline

Figure 1



Structure of most advanced 3CLpro inhibitors.

infectious peritonitis caused by a feline coronavirus [11[•],12–14]. GC-376 was confirmed to also block SARS-CoV-2 [15[•],16], providing a scientific rationale for a pre-IND submission for the treatment COVID-19 (<https://www.prnewswire.com/news-releases/an-ivive-repurposes-veterinary-drug-gc376-for-covid-19-and-submits-pre-ind-to-fda-301065619.html>). GC-376 is a sodium bisulfite prodrug of the aldehyde GC-373. Bisulfite adducts increase chemical stability and/or aqueous solubility of aldehydes. GC-373 has been described as a potent cathepsin B inhibitor [17], and its prodrug GC-376 has been described as a potent cathepsin L inhibitor [7]. The low target selectivity of GC-376 and many other 3CLpro inhibitors could present a challenge for clinical development. In particular, 3CLpro inhibitors that lack selectivity toward cathepsin L may potentially have counter-effective side effects by dampening the immune response against SARS-CoV-2 [18]. The broad spectrum of virus protease inhibition previously reported for GC-376 combined with the cathepsin L inhibition effect suggest its activity may extend to other human proteases. CDI-45205 (undisclosed structure) is the latest compound to advance into late preclinical stage (<https://www.cocrystalpharma.com/news/press-releases/detail/105/cocrystal-pharma-selects-lead-compound-for-further>). It was selected by Cocrystal Pharma at the end of 2020 from the broad-spectrum protease inhibitors discovered by Kansas State University Research Foundation, which demonstrated *in vitro* and *in vivo* activity in animal models against MERS and *in vitro* activity against SARS-CoV-2 [19].

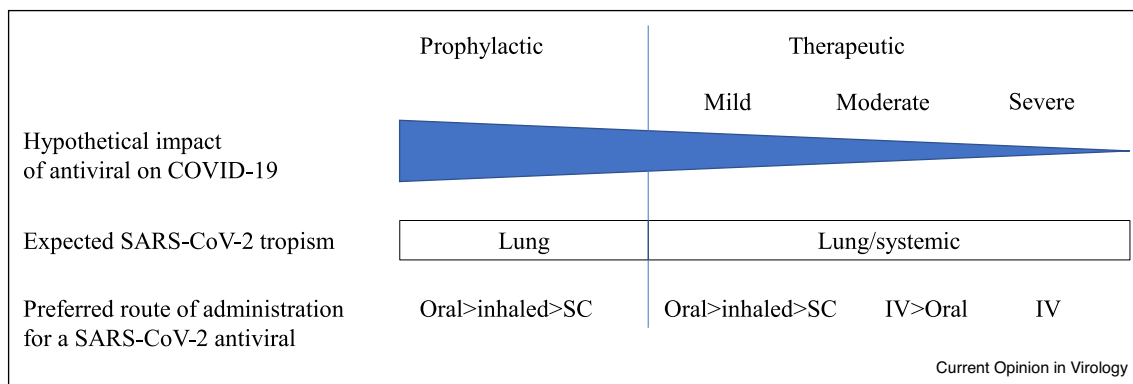
Routes of administration of first-generation inhibitors and emergence of a second-generation oral drug candidate

Although SARS-CoV-2 infects mainly the respiratory tract (pharynx, trachea, lungs) at the early stages of the self-limiting infection, a broader organotropism has been reported in more severe and advanced cases of illness [20–22]. In addition to the principal target organs of virus replication, several other factors need to be considered when optimizing the route of

administration of a 3CLpro inhibitor, including the timing of intervention relative to SARS-CoV-2 infection, COVID-19 disease stage, and bioavailability of the antiviral agent (Figure 2). The nucleotide analog remdesivir is approved in the United States for the intravenous infusion treatment of hospitalized patients only; therefore, the need for easier administration of a direct acting agent in early stage non-hospitalized patients remains high, as is the need for pre-exposure or post-exposure prophylaxis options. In this context oral delivery would be ideal for use in the early stages of disease management or in a pre-exposure or post-exposure setting. The intrinsically low oral bioavailability of the first-generation 3CLpro inhibitors has been a challenge to their clinical development. Both PF-00835231 and GC-376 have low oral bioavailability in rats of 1.4% and 3%, respectively ([10^{••}], Patent WO2013049382). The low oral bioavailability and short predicted half-life in human of PF-00835231 was improved with a highly soluble prodrug PF-07304814 that allowed continuous infusion using a minimal dosing volume to reach estimated minimal efficacious levels in clinical trials [10^{••}]. The planned route of administration for CDI-45205 is injection or inhalation for potential use as both a therapeutic and prophylactic (<https://www.cocrystalpharma.com/news/press-releases/detail/105/cocrystal-pharma-selects-lead-compound-for-further>). Other less advanced 3CLpro inhibitors have also been administered by subcutaneous or intraperitoneal injection to circumvent the issue of low oral bioavailability. For example, a SARS-CoV-2 3CLpro inhibitor (designated **11a**) had a bioavailability of 88% when dosed by intraperitoneal route in mice [23]. Separate reports that 3C-protease human rhinovirus peptidomimetic inhibitors have oral bioavailability greater than 20% in rodents and other nonclinical species suggest that this chemical class is potentially amenable to oral administration [6,24,25].

At the time of the publishing of this manuscript, Pfizer announced the launch of a Phase 1 clinical study (NCT04756531) with the second-generation orally available 3CLpro inhibitor PF-07321332 (<https://cen.acs.org/acs-news/acs-meeting-news/Pfizer-unveils-oral-SARS->

Figure 2



Considerations on the route of administration of a 3CLpro inhibitor. To be used in prophylaxis or early stage SARS-CoV-2 infection in outpatient setting, oral administration of a 3CLpro inhibitor is preferred over inhalation and subcutaneous (SC) injection. For more severe cases of COVID-19 requiring hospitalization, intravenous (IV) administration is preferred over oral and inhalation.

CoV/99/i13). PF-07321332 contains a nitrile warhead and was optimized for oral delivery by the reduction of the number of H-bond donors and application of a trifluoroacetyl capping group in P4 (Figure 1).

Pitfalls and challenges in assessing the preclinical antiviral potency of 3CLpro inhibitors

The need to discover and develop second-generation 3CLpro inhibitors with improved potency and/or bioavailability is high. In addition, 3CLpro inhibitors commonly target host proteases, which presents a potential liability for unexpected side effects. Therefore, increased target specificity might be critical for future development. However, preclinical evaluation of antiviral potency remains challenging for this class of compounds. Conflicting results of protease inhibitor testing emerged during the early months of the COVID-19 pandemic due to non-standardized assay conditions among research laboratories. For example, the three drugs shikonin, disulfiram, and ebselen previously approved for other indications were reported to inhibit SARS-CoV-2 3CLpro in enzymatic and antiviral assays [26^{••}]. However, enzyme inhibition was only achieved in the absence of physiologically relevant reducing agents, and the reported antiviral effect in cells infected with SARS-CoV-2 could not be reproduced and was therefore suspected to be an indirect consequence of cell death [15[•]]. Similar lack of reproducibility in SARS-CoV-2 inhibition due to cytotoxicity artefacts have been reported with HIV protease inhibitors nelfinavir and atazanavir [27]. These issues highlight the importance of proper assay conditions and controls to ensure correct interpretation of *in vitro* protease inhibitor testing results. Additional challenges in assessing 3CLpro inhibition potency in infected cells arose from the role of host proteases in virus entry. SARS-CoV-2 uses human ACE2 as its entry receptor and human proteases as entry activators including cell surface

transmembrane protease/serine (TMPRSS) proteases, furin, cathepsins, plasmin, elastase, and trypsin (for reviews: Refs. [28,29]). Although the relevance of individual host proteases at the site of infection remains subject to debate, the consensus is that SARS-CoV-2 enters cells mainly through direct membrane fusion by TMPRSS2 protease activation [30^{••}]. In addition to the TMPRSS2-facilitated direct entry from the cell membrane, SARS-CoV-2 can also enter cells through the endosomal pathway, where spike proteins are proteolytically activated by the lysosomal cathepsin L and/or B proteases [31]. Therefore, optimizing and developing protease inhibitors for SARS-CoV-2 face distinctive challenges due to the multiple host proteases and their level of redundancy for viral entry. *In vitro*, the antiviral activity of protease inhibitors largely depends on the mechanism of SARS-CoV-2 cellular entry and is driven by the levels of host protease expression in the cells. For example, the rhinovirus inhibitor rupintrivir and the cathepsin inhibitor K11777 block SARS-CoV-2 replication in A549 lung epithelial cells, but their antiviral effect was greatly diminished when TMPRSS2 was overexpressed [32[•]]. These examples highlight the difficulty of establishing physiologically relevant antiviral assays due to the influence of host protease expression on the mechanism of virus entry. For these reasons, cellular assays risk overpredicting the true potential antiviral effect of broad-spectrum 3CLpro inhibitors that also target cathepsin L and/or other host proteases involved in virus entry [7].

Consequently, it is imperative to validate the protease target (host and/or viral) and the antiviral approach with animal models of virus infection. GC-376 is probably the most studied protease inhibitor in animal models among the current candidates for SARS-CoV-2 treatment. GC-376 administered subcutaneously twice daily resulted in partial to full recovery in laboratory and client-owned cats infected

with feline coronavirus [11*,14]. In a mouse model of SARS-CoV-2 infection, intranasal and combined intranasal + intramuscular treatment with GC-376 only achieved marginal reduction in viral load [33]. The antiviral effect of GC-376 was improved when combined with GS441524, the parent nucleoside of the remdesivir prodrug. The lack of clear antiviral effect of GC-376 monotherapy in this study might be due to insufficient drug exposure caused by its suboptimal pharmacokinetic properties. Very recently, more convincing animal model efficacy data has emerged with other 3CLpro inhibitors. In a mouse model of SARS-CoV-1 infection, PF-00835231 significantly reduced lung viral titers up to two days post-infection, also alleviated signs of disease such as weight loss and lung pathology [10**]. Other molecules derived from boceprevir or telaprevir also demonstrated antiviral activity in a mouse model of SARS-CoV-2 infection [34*]. Finally, ALG-097111 was reported as a potent and highly selective 3CLpro inhibitor with antiviral activity in a SARS-CoV-2 hamster model [35].

Conclusion

The two main classes of direct acting agents for the treatment of SARS-CoV-2 infection are polymerase (nsp12) and protease (3CLpro) inhibitors. The nucleotide analog remdesivir is already approved as a polymerase inhibitor for hospitalized COVID-19 patients, and MK-4482/EIDD-2801, also a polymerase inhibitor, is efficacious in animal models and is currently being evaluated in the clinic [36–38]. In comparison, the most advanced 3CLpro inhibitor PF-07304814 is still in early stage clinical trial evaluation as an intravenous infusion treatment and has not yet been reported to demonstrate antiviral activity in humans. The emergence of the orally bioavailable clinical candidate PF-07321332 will help to address early stage non-hospitalized patients as well as potential prophylaxis settings. Although more proof-of-concept work is needed to fully validate 3CLpro inhibitors, this class of compounds provides a promising avenue to treat coronavirus infections either as monotherapies or in combination with other antiviral agents.

Conflict of interest statement

The authors of this manuscript have the following competing interests: JD is current employee of Aligos Therapeutics, Inc., and KV is current employee of Aligos Belgium BV.

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- of special interest
- of outstanding interest

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