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A rational roadmap for SARS-CoV-2/COVID-19 pharmacotherapeutic research and development

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Abstract

In this review, we identify opportunities for drug discovery in the treatment of COVID-19 and in so doing, provide a rational roadmap whereby pharmacology and pharmacologists can mitigate against the global pandemic. We assess the scope for targetting key host and viral targets in the mid-term, by first screening these targets against drugs already licensed; an agenda for drug re-purposing, which should allow rapid translation to clinical trials. A simultaneous, multi-pronged approach using conventional drug discovery methodologies aimed at discovering novel chemical and biological means targetting a short-list of host and viral entities should extend the arsenal of anti-SARS-CoV-2 agents. This longer-term strategy would provide a deeper pool of drug choices for future-proofing against acquired drug resistance. Second, there will be further viral threats, which will inevitably evade existing vaccines. This will require a coherent therapeutic strategy which pharmacology and pharmacologists are best placed to provide.

Abbreviations

3CL_{pro}, 3C-like proteinase of the virus

ACE, angiotensin-converting enzyme

ACE2, angiotensin-converting enzyme 2

ADRP, ADP-ribose-1st-phosphatase

ARDS, acute respiratory distress syndrome

BPS, British Pharmacological Society

CARD, caspase activation and recruitment domain

CoV, coronavirus

E, Envelope protein of the virus
FRET, Förster Resonance Energy Transfer
GtoPdb, BPS/IUPHAR Guide to PHARMACOLOGY database
IUPHAR, International Union of Basic and Clinical Pharmacology
M, Membrane glycoprotein of the virus
MERS, Middle East respiratory syndrome
N, Nucleocapsid protein of the virus
nsp, non-structural protein of the virus
PAMP, pathogen-associated molecular pattern
PL_{pro}, papain-like proteinase of the virus
RBD, receptor binding domain
S, Spike glycoprotein of the virus
SADS, Swine Acute Diarrhoea Syndrome
SARS, severe acute respiratory syndrome
TM, transmembrane

Author contributions

The document was conceived in discussions among SPHA, JA, JD, EF, SDH, FLS, AP and MJS; it was initially drafted by SPHA and all the co-authors contributed text and checked the manuscript; all the authors read and agree to submission of the manuscript.

Conflict of Interests

None of the authors has a conflict of interest to declare.

Introduction

PubMed has already accumulated a vast repository of information on SARS-CoV-2/COVID-19, which increases on a daily basis (on 2020-03-23, there were 1369 hits for COVID-19; this number more than doubled in the space of two weeks, so that by 2020-04-06 there were 2780 hits in PubMed for COVID-19). Clearly, there is a need to summarise this information critically and prioritise the elements which are constructive and useful for each individual sector. This document suggests priorities for how drug discovery and development might be rationally focussed for the rapid identification and successful translation of therapeutic agents to treat COVID-19.

Given the urgency of the current situation, clearly initial drug discovery should focus on repurposing licensed drugs, as dosage and safety information are largely to hand. Unfortunately, there is controversy over proof of efficacy for essentially all the potential repurposed agents for which preliminary, and, in many cases, non-peer reviewed data has surfaced. Some of this controversy is addressed below but efforts are underway from both WHO and NIH to coordinate larger, higher powered and better controlled studies in an attempt to demonstrate efficacy unequivocally. As a 'second wave', *de novo* discovery focussing on novel agents may allow future refinement and capacity to treat patients who are unable to be treated by, or are unresponsive to, the repurposed agents, but it would be very unlikely to have these new drugs available to treat the current crisis.

The IUPHAR/BPS Guide to PHARMACOLOGY (GtoPdb) is an open-access database, developed by the International Union of Basic and Clinical Pharmacology (IUPHAR) and the British Pharmacological Society (BPS). It provides expert-curated descriptions of almost 3,000 human proteins and over 10,000 ligands, including more than 1,400 approved drugs. Management of the new resource is the responsibility of the Nomenclature and Standards Committee of IUPHAR (NC-IUPHAR), which acts as the scientific advisory and editorial board. The committee has an international network of over 700 expert volunteers organized into 60 subcommittees dealing with individual target families. The database is notably enhanced through the continued linking of relevant pharmacology with key immunological data types as part of the IUPHAR Guide to IMMUNOPHARMACOLOGY (supported by the Wellcome Trust) and by a major new extension, the IUPHAR/MMV Guide to Malaria PHARMACOLOGY (in partnership with the Medicines for Malaria Venture). The GtoPdb team centred at the University of Edinburgh have constructed a resource (Facenda *et al.*), which provides a precis of the current understanding about the virus and potential associated drug targets and drugs. As with the other databases, the emphasis of the curation process is on stringent provenancing of the information provided, although inevitably the current situation limits the capacity for triangulation of data.

Nomenclature

Sequencing analysis of the novel virus has identified a high level of similarity with the virus identified to cause the Severe Acute Respiratory Syndrome (SARS) outbreak in China in 2002/03/04, which was known as the SARS coronavirus or SARS-CoV. Provisionally named as 2019-nCoV, the virus has been renamed SARS-CoV-2 (Viruses, 2020). For the purposes of this document, the virus is described as SARS-CoV-2, while the infectious disease is named as COVID-19 (World Health Organization, 2020). One of the positive aspects of the emergence of SARS-CoV-2 and COVID-19 is the rapidity at which aspects like genome sequencing (for example, Lu *et al.*, 2020) and 3D structures (for example, Yan *et al.*, 2020) have been described.

The viral cycle and virally-encoded potential drug targets

For general reviews of the coronaviruses, see Masters, 2006; Fehr and Perlman, 2015; de Wit *et al.*, 2016; Zumla *et al.*, 2016; Cui *et al.*, 2019; Desforgues *et al.*, 2019; Song *et al.*, 2019. SARS-CoV-2 is a betacoronavirus; a lipid-enveloped, single-stranded, positive sense RNA virus. Other human coronaviruses include alphacoronaviruses, such as human coronavirus-229E (HCoV-229E), and betacoronaviruses, such as SARS-CoV and MERS-CoV (responsible for the Middle East respiratory syndrome) (for review, see Zumla *et al.*, 2016; Corman *et al.*, 2018; Pillaiyar *et al.*, 2020). More than 200 viral types have been associated with the common cold, of which 50% of infections are rhinovirus, but also include respiratory syncytial virus, influenza and coronaviruses, particularly HCoV-229E. Although HCoV-229E is regarded as ‘relatively benign’ since monocytes are much more resistant to infection, it does rapidly kill dendritic cells (Mesel-Lemoine *et al.*, 2012).

Classically, the viral lifecycle can be divided into six elements: cell attachment; cell entry; viral uncoating; nucleotide replication; viral assembly, and viral release (see **Figure 1**). Positive-stranded RNA viruses replicate in the cytoplasm of infected cells, in close contact with intracellular membranes. This organization allows a concentration of viral and host factors to enable virus production and to evade innate immune responses (reviewed by Yager and Konan, 2019).

The SARS-CoV-2 coronavirus 30 kb genome encodes 29 proteins (Gordon *et al.*, 2020). Historically, therapeutic benefit has been gained through exploitation of the differences between viral and host proteins that subserve superficially similar functions (proteases and nucleotide polymerases, for example). The rapidity with which structural elements of the SARS-CoV-2 proteome have been identified provides hope that drug discovery approaches will soon provide agents to target the virus selectively, with minimal impact on the host. Based on the evidence from orthologous proteins from other betacoronaviruses and the information currently available on SARS-CoV-2 (some of it not yet from peer-reviewed sources), we propose here the priority targets for pharmacological investigation. That should not be taken to mean that research should

be limited to these targets, since there are undoubtedly a number of functions of the viral proteins still to be ascertained. It would be remiss not to conduct a thorough examination of all the viral proteome, both in isolation and in combination. The strategies we learn from investigation of the host:viral interaction from SARS-CoV-2 will stand us in good stead for future viral threats.

Cellular attachment and entry; replication, assembly and release

Coronavirus binds to cell surface proteins on target cells and, following proteinase priming of spike proteins on the virus surface, the virus is internalized into endosomal fractions that are subsequently acidified, or accumulates through a non-endosomal route (Fehr and Perlman, 2015). The endosomal route appears to involve clathrin (Inoue *et al.*, 2007), but there are contradictory reports of the importance of the intracellular C-terminus of ACE2 in this mechanism (see below) (Inoue *et al.*, 2007; Haga *et al.*, 2008). A fusion domain permits insertion of a key protein (Spike, see below), which then allows mixing of the viral and cellular membranes and subsequent release of the coronaviral genome into the cytoplasm.

Following entry into the host cell cytoplasm, the replicase gene of the viral RNA is translated. The genome of coronaviruses consists of a single, continuous, linear, ssRNA, capped at the 5' end and with a 3'-polyA tail (Fehr and Perlman, 2015). Translation occurs from open reading frame (ORF) 1a and 1b at the 5' terminus, with a ribosomal frameshifting mechanism allowing the overlap between ORF1a and ORF1b to generate the two polyproteins pp1a and pp1ab (Fehr and Perlman, 2015; Perlman and Netland, 2009; Snijder *et al.*, 2003; Thiel *et al.*, 2003). In SARS-CoV-2, the polyproteins are long, 4405 and 7096 aa, respectively. Encoded within the polyproteins of betacoronaviruses are two proteinases: papain-like proteinase, PL_{pro}, and chymotrypsin-like proteinase, 3CL_{pro}. In SARS-CoV, PL_{pro} has three endoproteinase target sites, which release non-structural proteins 1-3 (Thiel *et al.*, 2003). 3CL_{pro} has 11 cleavage sites to release the remaining non-structural proteins. In the family, these proteinases process the polyproteins to generate 16 functional non-structural proteins identified as nsp1-16 (Anand *et al.*, 2003; Thiel *et al.*, 2003; Ziebuhr *et al.*, 2007; Kindler *et al.*, 2016; Cui *et al.*, 2019).

Downstream of the ORF1a and 1b are genes encoding four structural proteins (Spike, Envelope, Membrane and Nucleocapsid) (see **Figure 2**) and a short series (described as at least 13 in total, Srinivasan *et al.*, 2020) of other proteins (see below). Once sufficient protein and RNA accumulate, coronavirus assembly takes place, centred on the structural proteins. The release of coronavirus particles involves the secretory pathway of the endoplasmic reticulum and Golgi apparatus and vesicular exocytosis (for review, see de Haan and Rottier, 2005; Fehr and Perlman, 2015), and it is likely, but as yet unconfirmed, that SARS-CoV-2 adopts this mechanism also.

To date, there is more evidence about the molecular detail involved in (and the possibilities to influence) viral recognition, entry and replication compared to uncoating, assembly and release, hence the attention paid here to the former three mechanisms.

Targeting virus recognition and cellular entry

The cell-surface anchor - ACE2

Among the coronaviruses, the spike protein interacts with proteinases to anchor on host cell surfaces. The cell-surface anchoring point for the alphacoronavirus HCoV-229E is aminopeptidase N (also known as CD13, Yeager *et al.*, 1992). For the betacoronavirus MERS-CoV, dipeptidylpeptidase 4 (also known as CD26, Raj *et al.*, 2013) is an anchor. Analysis of the co-crystal structure suggested that the SARS spike protein binds to the active site of angiotensin converting enzyme 2 (ACE2, Li *et al.*, 2005). Binding of SARS-CoV spike to ACE2 seems to require cholesterol-rich rafts in the host cells (Glende *et al.*, 2008). Recent evidence points to the spike protein of SARS-CoV-2 also binding to ACE2. Both SARS-CoV (Li *et al.*, 2003) and SARS-CoV-2 (Hoffmann *et al.*, 2020; Letko *et al.*, 2020) have been described to require ACE2 to enter cells. A particular domain of the spike protein of SARS-CoV-2, a so-called Receptor-Binding Domain (RBD), has been shown to facilitate binding to ACE2 (Hoffmann *et al.*, 2020). The ACE2 peptidase active site is

located remotely from the cell membrane (Li *et al.* , 2005; Wrapp *et al.* , 2020; Yan *et al.* , 2020), into which the Spike protein binds. The RBD of the Spike protein is located in the S1 ectodomain, approximately a third of the way along the protein. ACE2 is a carboxypeptidase, which means it removes the terminal amino acid from the C-terminus of oligopeptides, and so it seems unlikely that the Spike protein is a substrate for ACE2.

In SARS-CoV-infected mouse lung, ACE2 protein expression was downregulated compared to uninfected mice (Kuba *et al.* , 2005). Following SARS-CoV Spike protein administration to mice, angiotensin II was increased in the lungs (Kuba *et al.* , 2005). These observations led to the suggestion that this was the molecular mechanism for the frequent development of acute respiratory distress syndrome (ARDS) during SARS-CoV infections (Imai *et al.* , 2005; Kuba *et al.* , 2005).

ACE2 activity has been reported to be released from plasma membranes by proteolysis, thought to be through the action of TNF α convertase (ADAM17, A Disintegrin And Metalloproteinase domain containing protein 17, Lambert *et al.* , 2005). ACE2, and ACE, activity can be measured in human plasma (Ocaranza *et al.* , 2006; Herath *et al.* , 2007; Lew *et al.* , 2008). Human plasma ACE2 activity is reported to be ‘masked’ by the presence of endogenous inhibitors (Lew *et al.* , 2008), which don’t yet appear to have been precisely defined. Blood ACE2 activity can be altered in pathology; for example, serum ACE2 was found to be decreased in patients following acute ischemic stroke (Bennion *et al.* , 2016).

The expression of ACE2 mRNA and enzyme activity in cardiac tissues were increased following repeated oral administration of the AT₁angiotensin II receptor antagonist losartan, while oral administration of an ACE inhibitor lisinopril only increased cardiac mRNA expression, but not enzyme activity (Ferrario *et al.* , 2005).

Studies using disruption of the *ace2* gene in mice indicated an increase in circulating angiotensin II levels and a severe cardiac contractility defect, which could be ‘rescued’ with simultaneous genetic disruption of ACE (Crackower *et al.* , 2002). An early investigation of *ACE2* polymorphisms in man failed to show an association with hypertension (Benjafeld *et al.* , 2004) and a study of SARS victims and *ACE2* polymorphisms failed to find a correlation with patient outcomes (Chiu *et al.* , 2004).

The coronaviral Spike protein

The spike protein is the largest viral structural protein (~1200-1400 aa) and is heavily glycosylated, forming extended trimeric structures providing the characteristic ‘crown’ feature of coronaviruses (Belouzard *et al.* , 2012) (see **Figure 2**). The ectodomain is divided into the S1 domain responsible for binding to ACE2, whereas the S2 domain is responsible for the fusion machinery. Following binding of the S1 domain to ACE2, a deformation of the pre-fusion trimer results (Wrapp *et al.* , 2020). Surface plasmon resonance of the binding of human ACE2 to the immobilized SARS-CoV-2 indicated an affinity (K_d value) of 15 nM, an order of magnitude larger than SARS-CoV binding to ACE2 (Wrapp *et al.* , 2020). Using a related label-free technique, biolayer interferometry, affinities of 5 and 1.2 nM for binding of SARS-CoV and SARS-CoV-2 spike protein, respectively, to human ACE2 has been reported (Walls *et al.* , 2020).

Although a proteolytic cleavage site at the S1/S2 boundary of the SARS-CoV Spike protein is the best characterised, a second site upstream of the fusion peptide in the S2 domain, called S2’ has also been described (Belouzard *et al.* , 2009). This raises the possibility that multiple other proteases might be targeted to influence coronavirus activation (Millet and Whittaker, 2015).

The SARS-CoV S2 domain has a pair of α -helices, which may participate in coiled:coil structures during membrane fusion (Petit *et al.* , 2005). The host complex of ZDHHC9 (Link to UniProt) with GOLGA7 (Link to UniProt), a palmitoyltransferase, which modifies the low molecular weight G proteins NRAS and HRAS (Swarthout *et al.* , 2005), also palmitoylates the cysteine-rich S2 endodomain of the SARS-CoV to facilitate membrane fusion (Petit *et al.* , 2007).

Very recently, in a comparison of the S2 domains of SARS-CoV and SARS-Cov-2, an enhanced capacity of

the novel virus' S2 domain for membrane fusion was observed and suggested to result from eight differing amino acids (Xia *et al.* , 2020). Using a series of oligopeptides conjugated to lipid entities, high affinity (IC₅₀ values in the nanomolar range) inhibitors of cell fusion were identified.

Interfering with the ACE2:Spike interaction

Given that the spike protein binds to the active site of ACE2 (Li *et al.* , 2005), in theory, any alteration in the availability of the active site should influence the binding of the spike protein and, hence, interfere with SARS-CoV-2 infection. One option would be to provide an excess of an endogenous peptide substrate, or more conventionally to apply a selective enzyme inhibitor.

Endogenous substrates of ACE2

ACE2, discovered in 2000 (Donoghue *et al.* , 2000), shares 40% sequence similarity to ACE within the N-terminal domain and is a type I transmembrane metalloproteinase. Unlike ACE, it functions as a zinc carboxypeptidase to cleave single C-terminal amino acids from peptides, particularly hydrolysing Pro-Phe residues in angiotensin-(1-8) to angiotensin-(1-7), [Pyr¹]-apelin 13 to [Pyr¹]-apelin-(1-12) and [des-Arg⁹]-bradykinin to bradykinin-(1-8) with high efficiency. It may also cleave other peptides less effectively (Vickers *et al.* , 2002), shown below:

Angiotensin I	angiotensin-(1-9) + Leu
Angiotensin II	angiotensin-(1-7) + Phe
Apelin-(1-13)	QRPRLSHKGPMP + Phe
Apelin-(1-36)	...QRPRLSHKGPMP + Phe
[Des-Arg ⁹]-Bradykinin	RPPGFSP + Phe
Dynorphin A-(1-13)	YGGFLRRIRPKL + Lys

115 other peptides were not hydrolysed by ACE2 including adrenocorticotrophic hormone, calcitonin, cholecystokinin, met-enkephalin, glucagon, glucagon-like peptide-1, melanin-concentrating hormone, pituitary adenylyl cyclase-activating polypeptide, somatostatin-14, urocortin or vasoactive polypeptide (Vickers *et al.* , 2002).

In humans, levels of mRNA encoding ACE2, together with immunoreactive peptide, are highest in the gastrointestinal tract, followed by heart, kidney, testes and gall bladder and other tissues (Uhlen *et al.* , 2015). Within organs, ACE2 immunoreactivity was predominantly localised to epithelial (for example, in the lungs) and endothelial cells from all vascular beds examined (Yang *et al.* , 2017). Importantly, the ACE2 antisera used in this study for immunocytochemistry was the same as that employed in the study described in the section below "Using biopharmaceutical/antibody approaches to target ACE2:Spike interactions" (Hoffmann *et al.* , 2020), to block entry of the virus in cell culture. The epitope for this antisera would be a rational starting point for the development of selective therapeutic antibodies.

The presence of ACE2 on airway epithelial cells is consistent with the isolation of SARS-CoV-2 from bronchoalveolar lavage of patients with COVID-19 and the infection of cultured airway epithelial cells (Zhu *et al.* , 2020). In humans, levels of ACE2 immunoreactivity tends to be low. However, in addition to being upregulated by ACE inhibitors and angiotensin receptor antagonists (see above), ACE2 expression has been reported to be increased in human cardiovascular disease, for example, in the cardiomyopathic heart (Zisman *et al.* , 2003). Since ACE2 is critical for viral entry, it may be one explanation for the high incidence of co-morbidity of COVID-19 patients with cardiovascular disease.

Manipulation of ACE2 activity by synthetic agents

Assays employing fluorogenic surrogate substrates to screen for inhibitors of ACE2 activity are well-established, for example using methoxycoumarin-RPPGFSAFK(Dnp)-OH (Ocaranza *et al.* , 2006; Bennion *et al.* , 2016),

or methoxycoumarin-APK(Dnp)OH (Herath *et al.* , 2007; Lew *et al.* , 2008; Mores *et al.* , 2008). Detailed protocols for the use of methoxycoumarin-APK(Dnp)OH have been described for FRET-based high throughput screening (Sriramula *et al.* , 2017; Xiao and Burns, 2017).

This style of assay identified that ACE2 was not inhibited in the presence of 10 μ M lisinopril, enalaprilat, or captopril, inhibitors of angiotensin-converting enzyme (Tipnis *et al.* , 2000), and there are no licensed drugs described to inhibit ACE2 activity. However, DX600 is a peptide-based ACE2 inhibitor (Huang *et al.* , 2003), while MLN4760 and compound 28 are described as sub-nanomolar potency ACE2 inhibitors (Mores *et al.* , 2008).

There is evidence for allosteric regulation of ACE2 activity, in that a xanthenone derivative (XNT) was observed to **enhance** ACE2, but not ACE, activity *in vitro* with a potency of 20 μ M (Hernandez Prada *et al.* , 2008). *An in silico* study later identified a binding site in an allosteric hinge region of ACE2, distinct from the proteinase active site, against which 1217 FDA-approved drugs were screened (Kulemina and Ostrov, 2011). A subsequent kinetic assay with the recombinant enzyme and a fluorogenic substrate identified labetalol and diminazene as agents able to double the maximal velocity of ACE2 enzyme activity.

Whether any of these compounds alter the binding of the spike protein from either SARS-CoV or SARS-CoV-2 or viral infection in general does not appear to have been examined yet.

A further speculative area that should be explored further is the concept of enhancing the activity of the serine proteinase ADAM17 to increase cleavage and release of membrane bound ACE2. Peptides such as angiotensin II are reported in animal models to cause release ('shedding') following binding to AT₁ receptors (Xu *et al.* , 2017). Although angiotensin II is licensed by the Federal Drug Administration to treat sepsis (known as Giapreza, Davenport *et al.* , 2020), it would be inadvisable as a treatment for COVID-19 given the detrimental action of angiotensin II on the lungs. In contrast, the investigational agent [Pyr¹]-apelin-13 is currently used in clinical studies (Davenport *et al.* , 2020) and may also interact with its cognate receptor to downregulate membrane expressed ACE2. This peptide also has beneficial effects on the heart, including an increase in cardiac output (Japp *et al.* , 2010).

Using biopharmaceutical/antibody approaches to target ACE2:Spike interactions

An alternative approach to the small molecule manipulation of the ACE2 enzyme would be to target the spike or ACE2 proteins with selective antibodies. Antibodies directed against ACE2 led to a reduction in SARS-CoV-2 virus entry into target cells (Hoffmann *et al.* , 2020), although this is likely to be some distance away from a therapeutic application.

A truncated version of human recombinant ACE2, lacking the transmembrane domain, mitigated against SARS-CoV infection of cells (Li *et al.* , 2003) and has been used in animal models to reduce symptoms of severe acute lung failure (Imai *et al.* , 2005), diabetic nephropathy (Oudit *et al.* , 2010) and cardiac hypertrophy and fibrosis (Zhong *et al.* , 2010). Treating COVID-19 victims with a soluble form of ACE2 (Batlle *et al.* , 2020) or a fusion protein of the spike-binding portion of ACE2 combined with the Fc portion of human IgG (Lei *et al.* , 2020) has been suggested.

Apeiron Biologics has approval to conduct a Phase II clinical trial of APN01 (human recombinant ACE2) for the treatment of COVID-19 in three European countries (Austria, Germany and Denmark) (NCT04335136). This recombinant version of ACE2 was originally licensed to GlaxoSmithKline and previously tested as GSK2586881 in a Phase 2 multicentre trial (NCT01597635) in patients with lung injury or ARDS, both features of SARS and MERS (and now COVID-19). The study tested the hypothesis that cleavage of angiotensin II (which causes lung injury - vasoconstriction, inflammation, fibrosis, vascular leak, and sodium absorption) to angiotensin-(1-7), would have counter regulatory beneficial action and reduce long term injury. GSK2586881 was well-tolerated in patients with ARDS, and the rapid modulation of peptides of the renin-angiotensin system demonstrated target engagement, in that levels of angiotensin II decreased rapidly whereas angiotensin-(1-7) levels increased and remained elevated for 48 h, although the study was not powered to detect changes in acute physiology or clinical outcomes (Khan *et al.* , 2017).

Sera from convalescent SARS-CoV patients prevented the cell entry of SARS-CoV-2 (Hoffmann *et al.* , 2020) and this approach has been used with some success in the SARS, MERS and COVID-19 outbreaks (for review, see Bloch *et al.* , 2020). The difficulty in identifying the precise molecular mechanism/s of convalescent sera action and issues with collection, standardization and scaling-up will be a challenge (Bloch *et al.* , 2020).

A bacterial equivalent of ACE2 (based on 3D structure rather than primary sequence) termed B38-CAP has been described, which is reported to reduce hypertension and limit cardiac dysfunction in an animal model (Minato *et al.* , 2020). Whether this agent might provide a decoy anchor to ‘chelate’ viral particles prior to infection has not been investigated.

The cell-surface priming mechanism - TMPRSS2

The *TMPRSS2* gene encodes a cell-surface proteinase (transmembrane serine protease 2, TMPRSS2) and is located at chromosomal locus 21q22.3 in close proximity to *ERG* , a gene encoding an ETS transcription factor (Link to UniProt, Paoloni-Giacobino *et al.* , 1997). (*ERG* fusion with *EWS* leads to Ewing’s sarcoma) Fusion of the *TMPRSS2* and *ERG* (or the related *ETV1*) genes has been reported to occur in the majority of prostate cancers and is suggested to lead to an androgen-dependent amplification of ETS-regulated genes (Tomlinson *et al.* , 2005). TMPRSS2 expression is androgen-regulated (Lin *et al.* , 1999; Chen *et al.* , 2019); it is expressed highly in prostate cancer (Lin *et al.* , 1999; Lucas *et al.* , 2008) (for review, see Tanabe and List, 2017) and loss of TMPRSS2 in the prostate is associated with reduced metastatic potential (Lucas *et al.* , 2014). In aggressive versions of prostate cancer, TMPRSS2 undergoes autocatalytic proteolysis at Arg²⁵⁵-Ile²⁵⁶ (Afar *et al.* , 2001), where the two chains may remain in combination due to interchain disulphide bridges (Chen *et al.* , 2010) or the catalytic moiety may be secreted (Chen *et al.* , 2010). In LNCaP human prostate cancer cells, the PPAR α agonist fenofibrate was able to mitigate against the androgen receptor agonist-evoked increase in TMPRSS2 expression (Zhao *et al.* , 2013).

Following binding of the coronavirus to ACE2, TMPRSS2 ‘primes’ the spike protein to facilitate entry of the virus into the target cell (Hoffmann *et al.* , 2020; Matsuyama *et al.* , 2020). TMPRSS2 is a single transmembrane domain protein with a calcium-binding LDL receptor class A domain and an extracellular serine proteinase domain, which appears to cleave substrates preferentially at basic residues (arg/lys) (Paoloni-Giacobino *et al.* , 1997). Pathogenesis of two strains of influenza virus has been reported to be markedly diminished by gene disruption of *tmprss2* in mice (Hatesuer *et al.* , 2013; Tarnow *et al.* , 2014), inferring that targeting this enzyme may have antiviral potential.

Interfering with the TMPRSS2:Spike interaction

Using immunohistochemical responses (Bertram *et al.* , 2012) and, very recently, using single nuclei and single cell RNA sequencing (Lukassen *et al.* , 2020), as yet not peer reviewed) of lung samples from otherwise healthy subjects, ACE2 and TMPRSS2 were shown to be co-expressed in human bronchial epithelial cells, which could be a nexus for primary infection.

By analogy with the previous consideration of ACE2 (above), alternatives to manipulate TMPRSS2 activity would be to provide endogenous substrates or synthetic inhibitors. However, the potential to make use of endogenous substrates seems limited. Thus, although TMPRSS2 has been described to hydrolyse and activate the cell-surface G protein-coupled receptor proteinase-activated receptor 2 (Wilson *et al.* , 2005), mice lacking *tmprss2* failed to display an overt phenotype (Kim *et al.* , 2006).

As with ACE2, there are no reports of licensed drugs which inhibit TMPRSS2 activity. Cbz-GGR-aminomethylcoumarin has been described as a surrogate fluorogenic substrate suitable for high-throughput screening (Paszti-Gere *et al.* , 2016), although it is also a substrate for other proteinases, such as chymotrypsin. I432, a 3-amidinophenylalanine, has been described as a high affinity selective inhibitor (compound 92, K_i of 0.9 nM) of TMPRSS2 (Meyer *et al.* , 2013). In IPEC-J2 pig jejunal epithelial cells, 10-50 μ M I432 reduced TMPRSS2-derived product in cell media (Paszti-Gere *et al.* , 2016).

In an investigation of SARS-CoV entry into HeLa cells expressing recombinant ACE2 and TMPRSS2, a number of serine proteinase inhibitors (benzamidine, aprotinin, gabexate, tosyl lysyl chloromethyl ketone and camostat) were tested (mostly) at 10 μ M for 30 min before exposure to pseudotyped viruses. Only camostat was effective at reducing viral entry (Kawase *et al.* , 2012), and further experiment suggested that 1 μ M camostat was also effective, but only when TMPRSS2 was expressed. At 10 and 50 μ M, camostat inhibited cell entry of the SARS-CoV and SARS-CoV-2 spike protein (Hoffmann *et al.* , 2020). A direct inhibition of TMPRSS2 activity appears not to have been reported for camostat.

Potential ancillary proteins for virus entry - B⁰AT1/SLC6A19 and B⁰AT3/SLC6A18

The SLC6 family of transporters includes the well-characterised NET, SERT and DAT monoamine transporters, as well as the less well-exploited neutral amino acid transporter subfamily. B⁰AT1/SLC6A19 and B⁰AT3/SLC6A18 allow sodium- and chloride-dependent accumulation of neutral, aliphatic amino acids at the apical membranes of epithelial cells in the small intestine (B⁰AT1/SLC6A19) and kidney (B⁰AT1/SLC6A19 and B⁰AT3/SLC6A18) (for review, see Broer and Gether, 2012). B⁰AT3/SLC6A18 is also highly expressed in the GI tract and gall bladder (Protein Atlas) and may play a role in the faecal:oral transmission of coronavirus (Yeo *et al.* , 2020). The cell-surface expression of these neutral amino acid transporters is dependent on co-expression of ACE2 (Kowalczyk *et al.* , 2008; Fairweather *et al.* , 2012), aminopeptidase N (CD-13, Fairweather *et al.* , 2012) or collectrin (an adaptor protein, which has high homology to the transmembrane region of ACE2, Camargo *et al.* , 2009, Link to UniProt), in an apparently tissue-dependent manner (Kuba *et al.* , 2010). A recent cryo-EM structure suggested that ACE2 and B⁰AT1/SLC6A19 form a heterodimer which pairs up through interfaces between the two ACE2 partners, with the RBD of SARS-CoV-2 spike protein binding to the peptidase active site of ACE2 (Yan *et al.* , 2020) suggesting that B⁰AT1/SLC6A19 may facilitate entry of the novel coronavirus.

Interfering with the neutral amino acid transporters

Assays for B⁰AT1/SLC6A19 and B⁰AT3/SLC6A18 tend to be traditional accumulation of amino acids that were labelled with ionising or stable isotopes. Recently, a primary screen using a fluorescence-based membrane potential-based assay was used and followed up with a stable isotope accumulation assay to identify a novel inhibitor, cinromide, which exhibited modest potency (0.3-0.4 μ M) for inhibiting B⁰AT1/SLC6A19 in cell-based assays (Danthi *et al.* , 2019).

Targeting viral uncoating and replication

Viral uncoating

Once inside the cell, the endosomal cysteine proteases cathepsin B and cathepsin L have been described to process SARS-CoV (Simmons *et al.* , 2005) and this appears to be maintained for SARS-CoV-2 (Hoffmann *et al.* , 2020) although the significance of such intracellular proteinase activity is unclear. Potent inhibitors for these two proteinases have been reported as pharmacological probes, but there are no licensed drugs identified to target them.

Following entry into the cell, many viruses accumulate in acidified lysosome-like vesicles, and so weak bases (including ammonium chloride and chloroquine) which target the lysosome have been used *in vitro* to target this mechanism. Ammonium chloride (at 20 mM) has been described as a non-specific inhibitor of viral replication *in vitro* , targeting viral uncoating (Mizzen *et al.* , 1985) and, at 50 mM, ammonium chloride inhibited cell entry of both SARS-CoV and SARS-CoV-2 (Hoffmann *et al.* , 2020). Chloroquine was also observed to reduce infection of L cells by the coronavirus mouse hepatitis virus 3 (Krzyżystyniak and Dupuy, 1984).

Viral replication

Following entry into the cell, the virus subverts nucleotide, protein, lipid and carbohydrate turnover of the host cell to produce multiple copies of itself. The viral RNA is translated into multiple proteins to produce the replication machinery. As protein translation from the viral genome occurs, the two polyproteins are the first to be synthesised, with the two intrinsic proteases able to cleave the polyproteins into their constituent products.

Targetting the viral proteinases

The low sequence similarities between mammalian and viral proteases has allowed successful drug targetting of viral diseases, including both HIV/AIDS and HCV/hepatitis C. The genome of SARS-CoV-2 contains two proteinases intrinsic to the polyproteins, PL_{pro} and 3CL_{pro}.

The papain-like proteinase, PL_{pro}

The more N-terminally-located PL_{pro} is the larger (~2000 aa) of the two proteins (for review, see Baez-Santos *et al.*, 2015; Lei *et al.*, 2018), and, in SARS-CoV, is a membrane-associated, polyfunctional entity (Harcourt *et al.*, 2004). Sequence modelling of SARS-CoV-2 PL_{pro} suggested the presence of 6TM domains towards the C terminus, with the majority of the protein extending into the cell cytoplasm (Angeletti *et al.*, 2020). In other coronaviruses, the enzyme is also capable of hydrolysing ubiquitin from protein substrates (Barretto *et al.*, 2005; Ratia *et al.*, 2006), as well as removing the ubiquitin-like protein interferon-stimulated gene 15 (ISG, Link to UniProt) from ISG-conjugated proteins (Yang *et al.*, 2014). Using the orthologous proteinase from the mouse hepatitis coronavirus, analysis of three distinct structural domains suggested that the papain-like proteinase domain coincided with the deubiquitylating and deISGylating functions (Chen *et al.*, 2015). In SARS-CoV, the PL_{pro} also contains an ADRP functional phosphatase domain directed at ADP-ribose-1"-phosphates, although the functional significance of the hydrolase activity may be less impactful than the capacity to bind ADP-ribose, at least for the enzyme from HCoV-229E (Putics *et al.*, 2005). This domain is thought to contribute to processing of the viral subgenomic RNAs and the suppression of the innate immune system through reducing interferon production (Lei *et al.*, 2018).

Investigating the peptidase activity of SARS-CoV PL_{pro} suggested a preference for larger proteins (ubiquitinated or ISGylated) rather than simpler fluorescent-tagged oligopeptide substrates (Lindner *et al.*, 2005; Lindner *et al.*, 2007; Ratia *et al.*, 2014; Baez-Santos *et al.*, 2014) making screening more complicated.

The chymotrypsin-like proteinase, 3CL_{pro}

The smaller proteinase from SARS-CoV-2 is 3CL_{pro} (sometimes called the main prote(in)ase, M_{pro}). *In silico* docking models of SARS-CoV-2 3CL_{pro} has led to suggestions that particular existing antiviral agents, including velpatasvir and ledipasvir (licensed agents for treating hepatitis C when combined with sofosbuvir in the UK), should be screened for functional activity (Chen *et al.*, 2020). A recent screen of ~10,000 compounds including approved drugs, candidate drugs and natural products used a substrate derived from the N-terminal autocleavage site of the SARS-CoV-2 3CL_{pro} which was modified (methylcoumarinylacetyl-AVLQSGFR-Lys(Dnp)-Lys-NH₂) to allow a FRET-based assay (Jin *et al.*, 2020). The same substrate was used in a screen of the equivalent enzyme from another coronavirus, HCoV-HKU1, which transferred to humans (Zhao *et al.*, 2008).

A number of inhibitors of the SARS-CoV 3CL_{pro} proteinase have been described (Lu *et al.*, 2006; Yang *et al.*, 2006; Goetz *et al.*, 2007), without progressing into the clinic. Recently, an *in silico* approach using orthologues of the SARS-CoV 3CL_{pro} from other coronaviruses and enteroviruses allowed production and testing in vitro of a series of α -ketamides (Zhang *et al.*, 2020). One compound (11r) exhibited submicromolar potency against SARS-CoV 3CL_{pro} in a cell-free FRET-based assay, and micromolar potency in a cell infection assay with SARS-CoV (Zhang *et al.*, 2020).

In a preliminary (not yet peer-reviewed) report, the SARS-CoV-2 3CL_{pro} expressed in HEK293 cells was

found to interact with histone deacetylase 2 (HDAC2) by affinity purification/mass spectrometry (Gordon *et al.* , 2020). A number of approved drugs target HDAC2 in the treatment of various T cell lymphomas, including romidepsin, belinostat, and vorinostat with nanomolar potency (Bradner *et al.* , 2010).

Targeting nucleotide turnover

A relatively large proportion of the viral genome is inevitably devoted to nucleotide turnover. For SARS-CoV-2, this includes nsp7/nsp8/nsp12 as an RNA-dependent RNA polymerase; nsp13 as a helicase; nsp10/nsp14 as an 3'-to-5' exonuclease complex; nsp15 as an endoribonuclease and nsp16 as a 2'-O-ribose methyltransferase.

Remdesivir (currently in clinical trials to treat COVID-19), is described as a non-selective inhibitor of multiple RNA viruses, and has shown some efficacy in MERS-CoV and SARS-CoV infection of monkeys (de Wit *et al.* , 2020). In *in vitro* investigations, the triphosphate analogue of remdesivir inhibited RNA synthesis of MERS-CoV RNA-dependent RNA polymerase (primarily nsp8/nsp12 complexes derived from co-expression in insect cells of a construct containing nsp5, nsp7, nsp8 and nsp12) with an IC₅₀ value of 32 nM when nucleotide levels were low, increasing to 690 nM at higher nucleotide concentrations (Gordon *et al.* , 2020). *In silico* modelling identified that remdesivir, as well as the approved antiviral drugs ribavirin, sofosbuvir and tenofovir could bind tightly to the active site of nsp12 from SARS-CoV-2, based on the crystal structure of SARS-CoV (Elfiky, 2020).

However, ribavirin alone had no significant effect in a clinical trial with SARS victims, although combination of ribavirin with lopinavir-ritonavir and corticosterone had lower rating of ARDS and death (for review, see Zunla *et al.* , 2016). In-depth analysis has not been completed with MERS patients, although an ongoing Phase 2 clinical trial for MERS using a combination therapy of lopinavir/ritonavir and interferon β 1b (Arabi *et al.* , 2020).

Nsp13 is a helicase, which enables unwinding of duplex RNA. The exoribonuclease activity of nsp 14 sets the coronaviruses apart (Snijder *et al.* , 2003), as the enzyme is suggested to remove damaging mutations from the genome (Eckerle *et al.* , 2010; Sevajol *et al.* , 2014). In other coronaviruses, the endoribonuclease nsp15 has some selectivity for hydrolysing polyU sequences (Hackbart *et al.* , 2020). This enables the virus to delay or minimise initiation of the innate immune system by hydrolysing negative sense polyU nucleotides, which activate the MDA5 system to evoke interferon production (discussed further below). Nsp16 is a methyltransferase, which uses S-adenosyl-L-methionine as a co-substrate to assist in cap formation (Decroly *et al.* , 2008).

Protein: protein interactions in recombinant expression

In a preliminary (not yet peer reviewed) report, a series of tagged recombinant proteins from SARS-CoV-2 were expressed in HEK293 cells and then protein partners were identified by affinity purification/mass spectrometry (Gordon *et al.* , 2020). For nsp12 (RNA-dependent RNA polymerase) and nsp14 (3'-5'-exonuclease) of SARS-CoV-2, interactions with receptor interacting protein kinase 1 (RIPK1) and inosine monophosphate dehydrogenase 2 (IMPDH2), respectively, were identified. For these two targets, there are established approved drugs. Thus, ponatinib, which is used to treat acute myelogenous leukemia or chronic myelogenous leukemia (Philadelphia chromosome), targets multiple protein kinases, inhibiting RIPK1 with an IC₅₀ value of 12 nM (Najjar *et al.* , 2015). Mycophenolic acid and ribavirin are IMPDH2 inhibitors with IC₅₀ values of 20 nM (Nelson *et al.* , 1990) and 1-3 μ M (Wittine *et al.* , 2012) ranges, respectively, with clinical uses in organ transplantation and antiviral therapy, respectively.

Reservations about the use of ribavirin have already been noted above. Mycophenolic acid as a monotherapy was examined in a MER-CoV-infected non-human primate model, where the authors concluded it actually worsened the condition (Chan *et al.* , 2015).

Nsp13 (helicase) and nsp15 (endoribonuclease) have been described to bind to centrosome-associated protein 250 (CEP250) and RNF41 (also known as NRDP1, Link to UniProt), respectively, in a preliminary report

of recombinant expression (Gordon *et al.* , 2020). CEP250 is suggested to influence centrosome cohesion during interphase (de Castro-Miro *et al.* , 2016) and to be elevated in peripheral T cell lymphomas (Cooper *et al.* , 2011). The functional relevance of nsp13 interaction with CEP250 is not yet clear. RNF41 is an E3 ubiquitin ligase, which polyubiquitinates myeloid differentiating primary response gene 88 (MyD88, link to UniProt), an adaptor protein for Toll-like receptors, which allows activation of TBK1 and IRF3 (see below) and thereby increases type I interferon production (Wang *et al.* , 2009).

Targetting phospholipid turnover

The lipid profile of viruses appears to be important in terms of viral entry into the cell, either as sites for anchoring or for endocytosis (for review, see Heaton and Randall, 2011; Mazzon and Mercer, 2014). Replication of SARS-CoV is reported to take place associated with the endoplasmic reticulum in ‘replicative organelles’ incorporating convoluted membranes and interconnected double-membrane vesicles, inferring a capacity for the virus to induce extensive reorganization of intracellular phospholipid membranes (Knoops *et al.* , 2008). Three non-structural proteins from SARS-CoV with transmembrane domains, nsp3 PL_{pro} (see above), nsp4 and nsp6 when co-expressed in model cells prompted the formation of these double-membrane vesicles (Angelini *et al.* , 2013), although it is unclear whether specific catalytic activities are necessary for this action.

The lipidome of influenza virus (also a positive strand RNA virus) consists of glycerophospholipids, sterols (mainly cholesterol) and sphingolipids with sphingolipids and cholesterol enriched compared to the host cell membrane (Gerl *et al.* , 2012), but there does not yet appear to be a parallel investigation of SARS-CoV.

Cytosolic phospholipase A₂ α , cPLA₂ α , hydrolyses phospholipid to produce lysophospholipids and free fatty acids. Using alphacoronavirus HCoV-229E-infected Huh-7 cells, inhibition of cPLA₂ α using pyrrolidine-2 at higher concentrations (20 μ M) evoked an inhibition of viral titre (Muller *et al.* , 2018). Arachidonoyl trifluoromethylketone, a non-selective inhibitor of multiple eicosanoid-metabolising enzymes including PLA₂ isoforms, also inhibited viral titres at higher concentrations (Muller *et al.* , 2018). Transmission electron microscopy suggested that cPLA₂ α inhibition reduced the density of double-membrane vesicles (Muller *et al.* , 2018). Analysis of lipid metabolites indicated that HCoV-229E-infected Huh-7 cells showed increases in levels of ceramides, lysophospholipids and phosphatidylglycerols, with decreases in phosphatidic acids (Muller *et al.* , 2018). 20 μ M Py-2 inhibited the elevations in lysophospholipids and phosphatidylglycerols, but not the ceramides. Intriguingly, some selectivity of the involvement of PLA₂ α was suggested as Py-2 also displayed antiviral activities against other members of the *Coronaviridae* (and *Togaviridae*) families, while members of the *Picornaviridae* family were not affected.

Although speculative, there is the possibility that some of the benefits of glucocorticoid administration in the clinic might be the up-regulation of annexins, and the subsequent binding and concealment of membrane phospholipid from further metabolism (for review, see Lemmon, 2008). While clearly some distance from a validated target, since phospholipids are an essential component of enveloped viral proliferation, targeting the host availability of key structural lipids, particularly sphingolipids, has been proposed to be a useful strategy in preventing propagation of enveloped human RNA viruses, including influenza, HIV and hepatitis C (Yager and Konan, 2019). Currently, however, assays to screen inhibitors of cPLA₂ α are relatively limited.

Targetting carbohydrate turnover

Given that a number of the viral proteins, including the two structural proteins Spike and Membrane, are glycoproteins, there is clearly a diversion of sugars from the host. It is unclear as yet, whether specific sugars are involved and whether specific host glycosyltransferases are involved and might, therefore, form further tractable targets for drug discovery.

The other viral structural proteins

The E envelope protein

The Envelope proteins of SARS-CoV, HCoV229E and MERS are small (<100 aa) single transmembrane domain proteins (see **Figure 2**) and constitute ion channels with selectivity for monovalent cations over monovalent anions (Wilson *et al.*, 2004; Zhang *et al.*, 2014) apparently forming homopentamers in model membranes (Pervushin *et al.*, 2009; Surya *et al.*, 2015). Infecting or transfecting the coronavirus E message into cells results in accumulation of protein in the Golgi region (Ruch and Machamer, 2012). Conserved cysteine residues proximal to the TM domain internally within the virus are palmitoylated (Lopez *et al.*, 2008), a post-translational modification suggested to allow an internal inflexion point in the protein (Ruch and Machamer, 2012).

Hexamethylene-amiloride has been described as an inhibitor of the HIV-1 virus Vpu ion channel (Ewart *et al.*, 2002) and to reduce virus proliferation in human macrophages in culture (Ewart *et al.*, 2004). Hexamethylene-amiloride, but not the clinically-used amiloride, inhibited the SARS-CoV envelope protein-associated ion channel activity when expressed in HEK293 cells (Pervushin *et al.*, 2009).

Amantadine has had multiple uses clinically, including in the therapy of Parkinson's disease (for review, see Vanle *et al.*, 2018). It has been used to treat influenza A infection through targeting the M2 ion channel (Pinto *et al.*, 1992; Wang *et al.*, 1993; Holsinger *et al.*, 1994), although it is no longer recommended in the UK or US because of drug resistance (for review, see Li *et al.*, 2015). Amantadine at higher concentrations (100 μ M) was found to inhibit the SARS-CoV E protein expressed in model membranes (Torres *et al.*, 2007).

SARS-CoV E protein was identified as being pro-apoptotic upon transfection into Vero E6 monkey epithelial cells, where it localized to both plasma membrane and punctate cytoplasmic locations (Chan *et al.*, 2009). Indeed, the SARS-CoV E protein's ion channel function has been linked to calcium entry into endoplasmic reticulum/Golgi membrane complexes and the subsequent activation of the NLRP3 inflammasome, leading to interleukin- β (IL-1 β) production (Nieto-Torres *et al.*, 2015).

siRNA targeting of the Envelope protein of SARS-CoV reduced virus release in culture media, without altering N and P gene expression in FRhK-4 monkey kidney epithelial cells (Lu *et al.*, 2006). A similar observation was reported for the ORF4a protein of HCoV229E (Zhang *et al.*, 2014). Infecting mice with SARS-CoV in which the E protein ion channel function was disrupted showed unchanged viral proliferation but reduced IL-1 β and oedema levels in the lungs and prompted better survival over 10 days post-infection (Nieto-Torres *et al.*, 2014).

In a preliminary (as yet, unreviewed) report, the E protein of SARS-CoV-2 has been reported to interact with BRD2/BRD4 BET family bromodomain kinases when expressed in HEK293 cells (Gordon *et al.*, 2020). JQ1 and RVX208 are BRD2/4 inhibitors with IC₅₀ values with 40-120 and 50-1800 nM ranges, respectively, but there are no reports of clinically approved agents acting as inhibitors.

The M membrane protein

The membrane protein is usually regarded as the most abundant protein in the coronavirus envelope (see **Figure 2**) and is of intermediate size in SARS-CoV-2 (222 aa). It is thought to assist in viral assembly by collating the other surface structural proteins (Ruch and Machamer, 2012).

The N nucleocapsid phosphoprotein

The N protein is of moderate size in SARS-CoV-2 (419 aa), highly basic and binds the viral RNA as a dimeric entity (Fan *et al.*, 2005) into nucleocapsids (see **Figure 2**), which afford protection for the viral genome, while also providing access for replication at appropriate times (for review, see McBride *et al.*, 2014). In a preliminary (not yet peer reviewed) report, the N protein of SARS-CoV-2 was tagged and expressed in HEK293 cells and then protein partners were identified by affinity purification/mass spectrometry (Gordon

et al., 2020). The N protein was suggested to interact with casein kinase 2 (CK2), La-related protein 1 (LARP1, Link to UniProt) and stress granule protein Ras GTPase-activating protein-binding protein 1 (G3BP1, Link to UniProt). CK2 phosphorylates a broad range of cellular targets, mostly in the nucleus, to regulate development and differentiation (for review, see Gotz and Montenarh, 2017). Although not in use clinically, two inhibitors are described to target CK2 with high affinity. Silmitasertib is a CK2 inhibitor with an IC_{50} value of 1 nM (Pierreet *al.*, 2011), while TMCB has a K_i value of 21 nM (Janeczko *et al.*, 2012). LARP1 is an RNA-binding protein, which releases RNA when phosphorylated by mTORC1 (Fonseca *et al.*, 2015; Hong *et al.*, 2017). LARP1 seems to preferentially bind 5'-terminal oligopyrimidines with an as-yet unclear cellular role (Philippe *et al.*, 2020). Of the three targets suggested to associate with SARS-CoV-2 N phosphoprotein, G3BP1 seems a relevant focus for therapy against COVID-19. G3BP1 regulates the innate immune response (Kim *et al.*, 2019; Liu *et al.*, 2019; Wisner *et al.*, 2019; Yang *et al.*, 2019) and stress granules reduce the replication of MERS-CoV (Nakagawa *et al.*, 2018), so there is a potential for targeted drug discovery.

Interactions with the host innate immune system

SARS-CoV produces proteins that interfere with interferon pathways (nsp1, nsp3, nsp16, ORF3b, ORF6, M and N proteins, Wong *et al.*, 2016) and NLRP3 inflammasome activators (E, ORF3a, ORF8b) which are closely related to orthologues found in SARS-CoV-2. Fung *et al.* (2020) have recently reviewed the molecular aspects whereby SARS-CoV and, by inference, SARS-CoV-2, evades immune surveillance, activates the inflammasome and causes pyroptosis. Other coronaviruses may give an indication as to how this is happening. HCoV-229E rapidly kills dendritic cells, while monocytes are much more resistant. The rapid invasion of, and replication in, dendritic cells kills them within a few hours of infection (Mesel-Lemoine *et al.*, 2012). Dendritic cells are sentinel cells in the respiratory tract, and plasmacytoid dendritic cells are a crucial antiviral defence via interferon production, and by modifying antibody production. Thus, these viruses can impair control of viral dissemination and the formation of long-lasting immune memory. Penetration of SARS-CoV-2 infection deep into the lungs, and eventually the alveolae, results in the 'cytokine storm' which accompanies pneumonia and lung fibrosis and is probably a major determinant of the necessity for intubation, and also mortality (Shi *et al.*, 2020). It is currently not known what specific factor/s differentiate the patients who develop this; although mortality among younger health workers may indicate that initial viral load may play a role. Immunological agents which can prevent or control the 'cytokine storm' could therefore have a major effect on necessity to intubate and mortality. Tocilizumab is a monoclonal antibody targeting interleukin-6 receptors, as a means to interfere with the effects of chronic autoimmune disorders, such as rheumatoid arthritis. The Chinese Clinical Trial Registry has two studies that are designed to evaluate tocilizumab efficacy in patients with severe COVID-19 pneumonia (Registration Numbers ChiCTR2000029765 and ChiCTR2000030442). Similarly, anakinra, which is a slightly modified recombinant version of an endogenous antagonist of interleukin-1 receptors, is being investigated in clinical trials in multiple locations in patients with COVID-19 infection (NCT04324021, NCT04330638 and NCT02735707).

It has been suggested that in stage III of COVID-19, a critical point with a high viral load and severe respiratory involvement, lungs of patients appear with 'ground-glass' patches in CT scans, while autopsy reports indicate that the lungs are filled with a 'clear liquid jelly' (Shi *et al.*, 2020; Xu *et al.*, 2020), similar to an observation in drowning victims. On the hypothesis that inflammation-driven hyaluronan production (via hyaluronan synthase 2, HAS2, Link to UniProt), and associated water retention may be critical; a recent study proposed therapy via administration of recombinant hyaluronidase or inhibitors of HAS2 (Shi *et al.*, 2020).

The interaction between the virus and the innate immune system is complex and multifactorial, with temporal intricacies. It is beyond the scope of this review to identify all the multiple components and so we discuss here those pathways we consider most tractable.

Viral nucleotides and MDA5/MAVS/Interferon production

The positive sense RNA of coronaviruses is translated to produce the replication machinery, which allows complementary negative sense RNA to be synthesised, which itself is the template for the synthesis of positive strand RNA. As a consequence, double-stranded RNA is produced, which acts as a pathogen-associated molecular pattern (PAMP) targetting MDA5 (interferon induced with helicase C domain 1, also known as melanoma differentiation antigen 5, Kato *et al.* , 2006) from the RIG-1-like receptor family of cytoplasmic pattern recognition receptors (for reviews, see Schlee, 2013; Bryant *et al.* , 2015). MDA5 differs from RIG-1 (DexD/H-box helicase 58, also known as retinoic acid-inducible gene 1) in recognising longer dsRNA (Kato *et al.* , 2006; Goubau *et al.* , 2014), and it has been proposed this differentiates the sensing of positive-stranded viruses by MDA5 compared to negative strand virus sensing by RIG-I (Kato *et al.* , 2006; Goubau *et al.* , 2013). RIG-1-like receptors have an *N*-terminal caspase activation and recruitment domain (CARD), which shows ligand-dependent interaction with CARDS from other proteins, such as mitochondrial antiviral signalling protein (MAVS, Link to UniProt). MAVS activates IKK family kinases, such as TANK binding kinase (TBK1) and IKK- ϵ , leading to the phosphorylation of interferon regulatory factors, such as IRF3 (Link to UniProt) and IRF7 (Link to UniProt). This induces the transcription of Type I interferon genes, such as interferon- β and CCL5 (also known as RANTES) (Doyle *et al.* , 2002; Fitzgerald *et al.* , 2003; Sharma *et al.* , 2003). MAVS present in peroxisomes is also able to recruit short-acting, interferon-independent defense factors (Dixit *et al.* , 2010).

A number of other coronavirus proteins have been identified to influence the IRF3 pathway to restrict interferon production. This includes the MERS-CoV PL_{pro} proteinase (Yang *et al.* , 2014), as well as the ORF6 and Nucleocapsid proteins from SARS-CoV (Kopecky-Bromberg *et al.* , 2007). The Orf6 protein of SARS-CoV has also been described to reduce the activity of a series of karyopherin-dependent host transcription factors (Sims *et al.* , 2013). Karyopherin is an importin, which traffics proteins between the cytoplasm and the nucleus (for review, see Kosyna and Depping, 2018; Guo *et al.* , 2019).

Translocases of outer membrane 70 (Tom70, Link to UniProt) activates mitochondrial IRF3 (Liu *et al.* , 2010). The Orf9b protein of SARS-CoV-2 has been reported to interact with Tom70 when expressed in HEK293 cells (Gordon *et al.* , 2020).

The induction and suppression of interferon production have been extensively studied as they are central to numerous human diseases; the ‘trick’ to treat COVID-19 will be to identify a novel angle for therapeutic exploitation.

nsp1

Working with SARS-CoV (not SARS-CoV-2), Pfefferle and colleagues used yeast two-hybrid screens to identify interactions between the viral and human proteomes (Pfefferle *et al.* , 2011). They identified an interesting interaction between viral nsp1 and a group of host peptidyl-prolyl *cis-trans* -isomerases (PPIA, PPIG, PPIH and FKBP1A, FKBP1B), all of which modulate the calcineurin/NFAT pathway important in immune activation (reviewed by Hogan *et al.* , 2003). The nsp1 protein acts on these to activate NFAT signalling and immune activation. Cyclosporine A, an inhibitor of this pathway, has been used for several decades to control transplant rejection and some autoimmune diseases and, in a simple *in vitro* assay, cyclosporine inhibited SARS-CoV transcription/replication in (non-immune) cells (Pfefferle *et al.* , 2011). SARS-CoV-2 has an nsp1 closely related to that of SARS-CoV (Dong *et al.* , 2020; Srinivasan *et al.* , 2020), though its effect on the NFAT pathway seems not yet to have been reported. Nevertheless, cyclosporine has been shown to inhibit SARS-CoV-2 in an *in vitro* Vero cell-based assay in a preliminary report (as yet not peer-reviewed, Jeon *et al.* , 2020). It has therefore been suggested as a drug target (see, for example, Li and De Clercq, 2020). It may seem paradoxical to suggest an inhibitor of immune activation as a treatment for viral disease, but for the subgroup of patients that might suffer cytokine storms (Mehta *et al.* , 2020), the double-action might be useful.

ORF3a, ORF6, ORF8, Orf9c and other viral proteins

The ORF3a protein of SARS-CoV appears to bind calcium in a cytoplasmic domain (Minakshi *et al.* , 2014) and to elicit a response from the innate immune system by enhancing the ubiquitination of apoptosis-associated speck-like protein containing a CARD (Asc, Link to UniProt), which in turn activates the NLRP3 inflammasome and caspase 1 (Siu *et al.* , 2019). The potential for targeting Asc and the NLRP3 inflammasome for therapeutic benefit in inflammatory conditions has recently been reviewed (Mangan *et al.* , 2018), although there are no inhibitors in the clinic as yet.

In SARS-CoV, the *Orf8a* and *Orf8b* genes became separated, as the disease progressed, by a 29-nucleotide deletion (Chinese SARS Molecular Epidemiology Consortium, 2004; Oostra *et al.* , 2007). The *Orf8a* gene of SARS-CoV encodes a short (31 aa, 1 TM, Link to UniProt) protein, which forms a cation channel of predicted pentameric structure (Chen *et al.* , 2011). In SARS-CoV-2 and a bat-derived coronavirus, in contrast to the SARS-CoV-2 genome, *Orf8* which encodes a continuous 121 aa ORF8 protein (Cagliani *et al.* , 2020). Given that sequence analysis of different strains of SARS-CoV-2 suggests that the Orf8 locus displays only limited evidence of positive selection (Cagliani *et al.* , 2020), it seems germane to investigate the profile of ORF8 in more depth. Sequence comparisons led to prediction of secondary structure composed of an α -helix and a β -sheet containing six strands (Chan *et al.* , 2020), but there appears not to be any literature as to whether this entity is a functional ion channel.

In a preliminary (as yet, unreviewed) report, the Orf9c protein of SARS-CoV-2 has been reported to interact with NOD-like receptor X1 (NLRX1), proteinase-activated receptor 2 (PAR2/F2RL1) and NEDD4 family-interacting protein 2 (NDFIP2, *impdh2* Link to UniProt), among other proteins of the I κ B/NF κ B pathway, when expressed in HEK293 cells (Gordon *et al.* , 2020). At the moment, there are no approved drugs targeting PAR2, although AZ3451 (Link to GtoP) acts as a negative allosteric modulator with pIC₅₀ values of 5-23 nM (Cheng *et al.* , 2017).

There is a limited insight into the roles or potential exploitability of the remaining range of other viral proteins (nsp2; nsp9; nsp11, Orf3b; ORF6; ORF7a; ORF7b; ORF10).

Animal models of SARS-CoV-2 infection

The spike glycoproteins in SARS-CoV and MERS-CoV are crucial for host specificity and jumping between species, e.g. from bats to humans (Luet *et al.* , 2015), and from dromedary camels to humans (MERS-CoV) and also the recent cross-over of a HKU2-related coronavirus to pigs as a Swine Acute Diarrhoea Syndrome (SADS-CoV) (Zhou *et al.* , 2018). SADS-CoV appears to influence the innate immune system by reducing interferon- β production evoked through IPS-1 and RIG-I pathways (as described above), but not through IRF3, TBK1 and IKK ϵ (Zhou *et al.* , 2020).

ACE2, as the anchoring point for the Spike glycoprotein, is present throughout the animal kingdom, but small structural differences are critical in influencing this interaction with the coronavirus (Li *et al.* , 2020; Luan *et al.* , 2020). Key sequences of the Spike protein from SARS-CoV and SARS-CoV-2 are responsible for binding to ACE2. Luan *et al.* (2020) found that the key residues in the S protein from SARS-CoV and SARS-CoV-2, recognised by ACE2 from dog, cat, pangolin and *Circetidae* mammals (simulated through homology modelling) were broadly similar. Mouse ACE2 is inefficient in prompting entry of both SARS-CoV and SARS-CoV-2 (Fung *et al.* , 2020). Cats and dogs suffer from their own specific coronavirus infections (e.g. canine respiratory coronavirus, feline coronavirus) without significant cross-over to humans. A preliminary (as yet lacking peer review) report has suggested that cats and ferrets are sensitive to SARS-CoV-2, but dogs, pigs, chickens and ducks are much less sensitive (Shi *et al.* , 2020). Ferrets have been used as models for respiratory tract infections and retain the SARS-CoV-2 virus in the respiratory tract. Shi *et al.* (2020) showed that the infection was transmitted between cats by aerosol, which may have implications for confinement; infected cats subsequently produced antibodies.

The Syrian hamster has been used as a model for SARS-CoV (Robertset *et al.* , 2005; Roberts *et al.* , 2006; de

Wit *et al.*, 2013) and studies with mice and Syrian hamsters are ongoing with SARS-CoV-2. A preliminary report (as yet not peer-reviewed) suggests that monkeys can be infected and show signs of sickness similar to COVID-19, producing antibodies which minimize the signs of subsequent infection (Bao *et al.*, 2020).

Thus, while there is intensive research in animal models, a clearly validated model is still not apparent.

Inter-individual variations in susceptibility

Given the similarities in the viruses and their symptoms, there is clearly a value to comparing the profiles of sufferers from the original SARS, and subsequent MERS, outbreaks with COVID-19 to evaluate the risk factors associated with each event individually and collectively. A detailed consideration is beyond the scope of this review, but there are some obvious questions to ask (not in an order of priority).

1. What factor/s determine resistance to infection? It is apparent that many individuals who test positive for SARS-CoV-2 infection only experience ‘mild’ symptoms, others suffer a level of debilitation requiring hospitalization with limited supervision, and a third group require assisted breathing.
2. Is blood group a predictor? There is preliminary evidence (as yet, not peer-reviewed) suggesting that people with type A blood might be more at risk of COVID-19 than those with other blood types (Zhao *et al.*, 2020).
3. Are there ‘simple’ genetic markers which predict this variation? For example, are single nucleotide polymorphisms/haplotypes for key targets (including ACE2, TMPRSS2, etc, Delanghe *et al.*, 2020) associated with higher or lower damage in humans infected with SARS-CoV, MERS-CoV or SARS-CoV-2?
4. Reports suggest that there is a preponderance of male victims of COVID-19, for example in Spain (Instituto de Salud Carlos III, Ministry of Science & Innovation, Spain. Retrieved on 2020-03-25, referring to data from 2020-03-24). What might be the cause of this sexual divergence?
5. Is smoking history a predictor of variation? One potential explanation for the relatively high proportion of male victims has been suggested to be previous smoking history (Cai, 2020; Olds and Kabbani, 2020; Vardavas and Nikitara, 2020), clearly a general risk factor for many diseases. Is there evidence from the SARS and MERS outbreaks to suggest a commonality of susceptibility?
6. What is the impact of contracting the virus on individuals with other underlying conditions? For example, what are the mechanism/s underlying why some sufferers of hypertension and/or diabetes might be at higher risk (<https://www.immunopaedia.org.za/breaking-news/why-are-hypertension-and-diabetes-patients-at-high-risk-of-severe-covid-19/>)?
7. How will the evolution of the virus alter rates of infection and the severity of symptoms? Some level of mutation is to be expected, and indeed has been noted for the SARS-CoV-2. At the moment, it is too early to identify the significance of any influence of these mutations on the course of COVID-19.

Some of these questions are more tractable since the SARS and MERS outbreaks because of the strides being made in sophisticated molecular biological techniques (e.g. NextGen Sequencing). An additional distinction compared to the previous outbreaks is the major increase in patient numbers associated with COVID-19, allowing greater comparisons to be made in many more geographical locations.

Inevitably other questions will form as greater detail becomes available.

Conclusion and recommendations

This review has concentrated on the prevailing hypothesis that an essential first step in infection is SARS-CoV-2 binding to ACE2 and for TMPRSS2 to prime the viral Spike protein. We further hypothesise that both proteins must be expressed on a single target cell for the virus to gain entry. TMPRSS2 has an extensive cellular expression profile, whereas ACE2 is more limited and is usually at low levels, unless increased by risk factors such as being sex, age, and smoking history, so is likely to be rate-limiting. Other potential target proteins such as cathepsin L or B⁰AT1 may also prove important.

Currently, although there are no drugs approved for the treatment of patients with COVID-19, the pandemic has triggered a stampede into clinical trials with both approved and investigational agents. The pharmacological rationale for these trials is sometimes obscure, but there is a logic to focus on viral entry and replication, as well as limiting the host immune response.

For the immediate term, the highest priority would be to investigate known antivirals to mitigate effects of COVID-19. For the longer term, a vaccine (for review, see Amanat and Krammer, 2020) seems to hold the most promise to reduce COVID-19 damage. There is also a role in the mid-term, however, for drug discovery conducted in mainstream pharmacology labs. The goal here would be an international co-ordinated approach to drug re-purposing; examining the spectrum of licensed drugs (likely to be less than 2000, varying dependent on jurisdictions). These would ideally be screened in a co-ordinated, blinded fashion in multiple labs simultaneously to account for any minor methodological differences. This requires the re-opening of screening and protein biosynthesis labs closed at the start of the pandemic, while ensuring that workers are kept safe.

If one were to write a Target Product Profile for a drug to treat COVID-19, several parallel profiles can be identified. There are clear considerations, which may be identified as desirable pharmacodynamic, screening methodologies, drug metabolism and pharmacokinetic and formulation profiles.

From a pharmacodynamic perspective, a priority would be to screen the proteinases identified in this review (ACE2, TMPRSS2, ADAM17, cathepsin L, cathepsin B, PL_{pro} and 3CL_{pro}). A second parallel stream would assess inhibitors of the viral RNA polymerase and endoribonuclease complexes, as well as the ion channel functions of the viral Envelope (and potentially the Orf8 protein). Clearly, there are multiple other targets, which might bear fruit, and so further studies should assess the tractability of B⁰AT1/SLC6A19, B⁰AT3/SLC6A18, IMPDH2 and HAS2. Further, the molecular mechanism of action of ivermectin should be assessed, since it has recently been shown to inhibit *in vitro* SARS-CoV-2 replication (Caly *et al.*, 2020). This agent is used clinically as an anthelmintic, probably through blocking invertebrate glutamate receptors, although it also inhibits mammalian glycine receptors and acts as a positive allosteric modulator of other mammalian ligand-gated ion channels.

From a screening aspect, biophysical and biochemical screens would probably take a matter of days-to-weeks. Following mass availability of the recombinant proteins involved, the capacity for inhibition should be assessed using a library of already approved drugs. Biophysical methods can be applied, such as surface plasmon resonance or biolayer interferometry, to monitor the affinity of interaction between host ACE2 and viral spike glycoprotein in the presence of these agents, as well as the relevant proteins where multimerization is critical, such as the trimeric Spike glycoprotein. Assessing the remainder of the targets would likely adopt standard, fluorescent-based pharmacological methodologies.

A desirable element would also be to minimise adverse effects on the cardiovascular and respiratory system, given the high incidence of damage described associated with those systems (Esler and Esler, 2020; Li *et al.*, 2020; Lippi *et al.*, 2020). Candidate drugs should also not increase activity of the IL-6 (or any other pro-inflammatory cytokine) pathway to avoid provoking a cytokine storm.

If a similar approach were taken to the ways in which targetted therapy is applied for certain types of cancer, there would be an increased benefit in a multimodal strategy. Thus, in cancers where EGF/EGF receptors are involved, it is possible to target the ligand using chelating antibodies, to antagonise the receptor using blocking antibodies, to use specific antibodies to prevent dimerization of the receptor and to inhibit the catalytic activity of the receptor with small molecular inhibitors. It should be possible to reproduce this approach by simultaneously targetting several steps in the viral cycle (while naturally being cognisant of the potential for phenomena of drug:drug interactions, for instance in terms of convergent pathways of drug metabolism). This approach should also show benefit in reducing the capacity for drug-driven mutation in the enzyme.

From a DMPK perspective, a beneficial profile for any agent would avoid drug:drug interactions by not converging on key metabolic enzymes and/or transporters. Ideally, a once-daily treatment regimen would

be optimal, but if more frequent administration were needed, there is likely to be good patient adherence, given the public response to ‘spatial distancing’. From a formulation perspective, prophylactic use or for treatment of mild symptoms, an orally-administered or inhaled formulation would be appropriate. For more severe cases, where breathing is significantly impaired, an inhaled aerosolised version may be difficult to administer effectively; in this circumstance, a soluble version to be applied intravenously is likely to be useful.

Micro-organisms, such as viruses and bacteria, continue to evolve to evade our immune systems and previous pandemics contributed to the decline and fall of civilizations. HIV/AIDS became more widespread in the last century and was associated with high morbidity and mortality. As a result of the discovery of novel pharmacological treatments, including specific antivirals, it is now a chronic condition and a cure has been effected in at least two individuals. This gives us hope that the roadmap outlined in this review may provide some relief from COVID-19 (and indeed for viral threats yet to come).

Legend to Figures

Figure 1 : a graphical representation of the viral lifecycle, initiated at step 1 cell attachment and finishing with viral release.

Figure 2 : a cartoon of the virus structure, identifying the four structural proteins and the viral genome.

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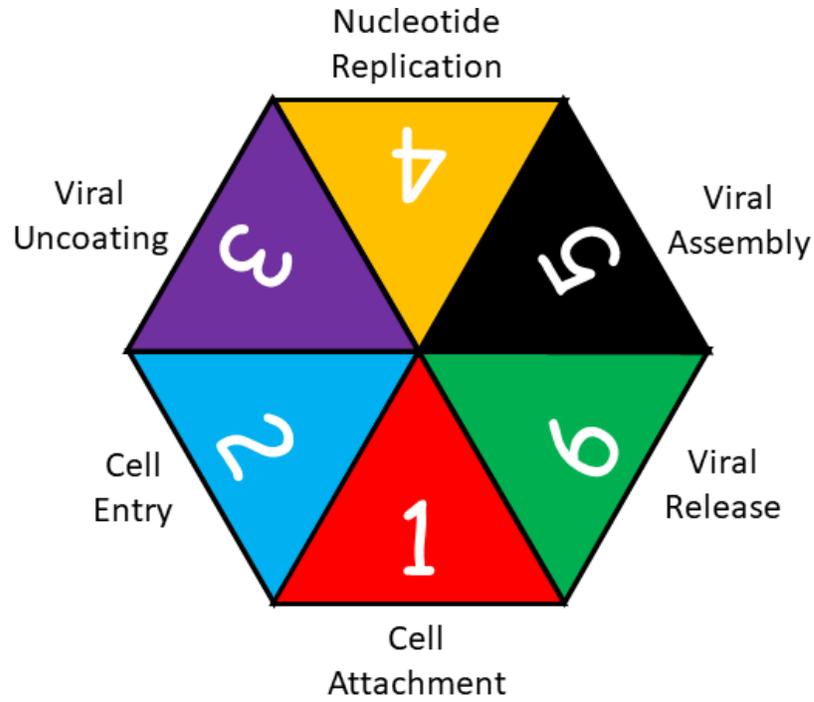


Figure 1: a graphical representation of the viral lifecycle, initiated at step 1 cell attachment and finishing with viral release.

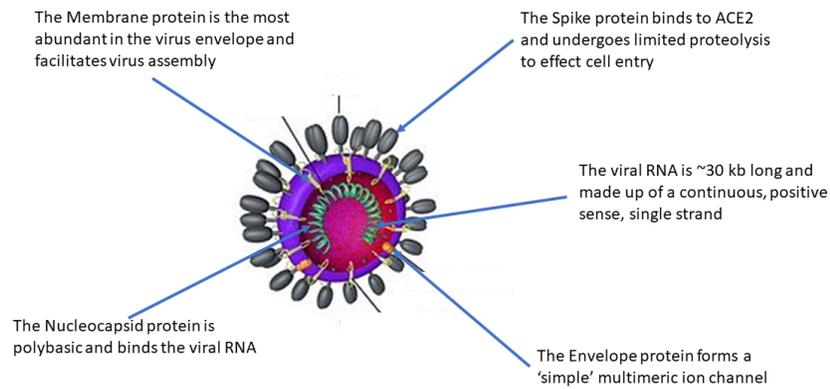


Figure 2: a cartoon of the virus structure, identifying the four structural proteins and the viral genome.

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