



Feature

Targeting viral replication complexes with mRNA-encoded nanobodies: a new frontier for antiviral design

Emerging and re-emerging RNA viruses continue to challenge global health preparedness, underscoring the need for broad-spectrum antivirals that can be rapidly deployed. We propose a family-specific antiviral design strategy that targets conserved replication–transcription complexes (RTCs) using nanobodies delivered as mRNA therapeutics. This approach overcomes the long-standing limitation of intracellular delivery of antibody-based biologics. By expressing antiviral nanobodies directly inside infected cells via lipid-nanoparticle-encapsulated mRNA, it becomes possible to disrupt essential protein–protein interactions within viral RTCs. Using SARS-CoV-2 non-structural protein 9 (NSP9) as a proof-of-concept, we show that stabilizing non-functional NSP9 oligomers can inhibit viral replication. This combined nanobody–mRNA technology provides a versatile platform for rapid antiviral development across virus families.

Keywords: nanobodies; mRNA therapeutics; SARS-CoV-2; replication transcription complex; coronavirus; antiviral; Pandemic preparedness

Introduction

The fundamental genomic characteristics of viruses were described by David Baltimore (Nobel prize in Physiology or Medicine, 1975), with a classification of animal viruses into six categories^(p1): (i) viruses that have double-stranded DNA (dsDNA); (ii) viruses that have a single-stranded positive DNA (+DNA); (iii) viruses that have a dsRNA genome; (iv) viruses that have a positive single-stranded RNA genome (+RNA); (v) viruses that have a negative single-stranded RNA genome (–RNA); and (vi) positive RNA tumor viruses that have a DNA intermediate. A

seventh category was later added to the Baltimore system that consists of a gapped DNA genome (vii), in which partially dsDNA must be filled to produce perfect duplexes. This system highlights two fundamental common features of all viruses: their ability to replicate viral genomic copies and to transcribe a viral genome into mRNA that can be read by the host translational machinery to produce viral proteins. RNA viruses must all encode for an RNA-dependent RNA polymerase (RdRp) enzyme able to accomplish both tasks: replication and transcription. The RdRp enzymes are highly conserved

within a family of RNA viruses and thus constitute ideal pan-family targets for the design of broad-spectrum antivirals.

Beyond their genome types, RNA viruses also encode essential cofactors necessary for their lifecycles, with roles in viral replication, transcription and virion assembly (Table 1). The timing and location of RdRp and cofactor actions are genome-dependent, with some polymerases packaged within the virion and others translated after infection. Besides RdRp, the cofactors provide additional therapeutic targets. For example, the *Arenaviridae* family is characterized by a

TABLE 1

Viral families of pandemic potential. Genome types, core polymerase enzymes, their cofactors and replication strategies are indicated. These family-specific replication mechanisms are proposed as antiviral targets.

 Viral Family	 Genome Type	 Polymerase (RdRp)	 Cofactors & Associated Proteins	 Replication Strategy
Filoviridae (e.g., Ebola, Marburg virus)	(–) ssRNA, non-segmented	L protein (RdRp)	VP35 (Cofactor), VP30 (transcription factor), NP (Nucleoprotein)	Uses transcriptional start-stop mechanism similar to Paramyxoviridae
Paramyxoviridae (e.g., Measles, Nipah, RSV)		L protein (RdRp)	P (Cofactor), N (Nucleoprotein), M (Matrix)	Uses start-stop transcription, replicates in cytoplasmic inclusion bodies
Arenaviridae (e.g., Lassa virus)	(–) ssRNA, segmented	L protein (RdRp, endonuclease)	NP (Nucleoprotein), Z (Matrix protein, inhibits RTC)	Replicates in the cytoplasm
Nairoviridae (e.g., Crimean-Congo hemorrhagic fever virus)		L protein (RdRp, endonuclease)	N (Nucleoprotein)	Replicates in Golgi-derived vesicles
Orthomyxoviridae (e.g., Influenza virus A, B, C, D)		PB1 (Polymerase), PB2 (Cap-binding), PA (Endonuclease)	NP (Nucleoprotein)	Replicates in the nucleus (unlike most RNA viruses)
Phenuiviridae (e.g., Rift Valley fever virus, Heartland virus)		L protein (RdRp, endonuclease)	N (Nucleoprotein)	Replicates in cytoplasmic vesicles
Hantaviridae (e.g., Hantavirus, Seoul virus)	(–) ssRNA, segmented (tri-segmented: S, M, L)	L protein (RdRp, endonuclease)	N (Nucleocapsid protein)	Replicates in cytoplasmic inclusion bodies
Coronaviridae (e.g., SARS-CoV-2, MERS-CoV)	(+) ssRNA, non-segmented	NSP12 (RdRp)	NSP7 & NSP8 (cofactors), NSP9 (RNA-binding), NSP13 (Helicase), NSP10/NSP16 (methyltransferase)	Uses subgenomic RNA strategy, replicates in double-membrane vesicles
Flaviviridae (e.g., Dengue, Zika, West Nile virus)		NS5 (RdRp + methyltransferase)	NS3 (Helicase), NS2B (Protease cofactor), NS4A/NS4B (Replication complex assembly)	Replication occurs in ER-derived replication factories
Togaviridae (e.g., Chikungunya, Sindbis virus)		NSP4 (RdRp)	NSP1 (Capping enzyme), NSP2 (Helicase/Protease), NSP3 (Scaffolding protein)	Uses subgenomic RNA strategy, replicates in cytoplasmic replication factories

matrix protein known as Z protein, which regulates viral RNA synthesis, inhibits polymerase activity in the late stages of infection and coordinates virion assembly and particle formation. Z protein is thus a promising target for pan-arenavirus inhibition.

Similarly, coronaviruses encode several cofactors that stabilize and regulate their replication–transcription complexes (RTCs), including non-structural proteins (NSPs) NSP9, NSP10, the helicase NSP13, the 3′-5′ exonuclease NSP14, the polyU-specific endoribonuclease NSP15 and the 2′-O-ribose methyltransferase NSP16. These proteins function alongside the core polymerase complex [NSP12-7-(8)₂] and offer multiple avenues for antiviral inter-

vention. In the *Filoviridae* family, VP30, VP35 and NP are polymerase-associated factors that also present valuable therapeutic targets (Table 1). We propose that, for each family of pandemic viruses, specific intracellular cofactors be identified as potential drug targets, enabling the design of inhibitors that could be used in combination therapies with polymerase inhibitors.

Previously, we outlined a pipeline for the systematic identification of host targets and the rapid design of broad-spectrum small-molecule antivirals.^(p2) This strategy stands to benefit significantly from evolving artificial intelligence (AI) tools such as AlphaFold, RoseTTAFold and their improved versions,^{(p3),(p4),(p5),(p6),(p7)} which

are expected to substantially expand the antiviral small molecules arsenal. However, complete mitigation of pandemic virus replication requires complementary strategies. To this end, we have introduced a novel approach that combines two powerful technologies: nanobodies and mRNA-LNP, as an innovative pathway to design family-specific biologic antivirals.^(p8)

Rationale for the use of nanobodies in drug design

In 1993, the laboratory of Prof. Hamers (Vrije Universiteit of Brussels, Belgium) serendipitously discovered ‘nanobodies’ in camelids.^(p9) Nanobodies are derived from heavy-chain-only antibodies

(HCAbs), consisting of a dimer of identical heavy chains (VH), as opposed to classical antibodies that also possess light chains (VL). The HCAb variable heavy chains lack CH1 domains and exhibit several amino acid changes (with respect to the corresponding highly conserved domains in normal antibodies) to allow pairing.^{(p9),(p10)} The variable domains of the two HCAb components are known as variable domains of heavy chains of HCAbs (VHHs) and, importantly, can be manipulated using conventional DNA engineering technologies.^{(p11),(p12)} Compared with monoclonal antibodies, nanobodies have attractive properties such as small sizes (15 kDa vs 50 kDa) enabling tissue penetration, higher affinity, access to 'hidden' epitopes, stability and reversible unfolding, solubility and ease of production.^{(p13),(p14),(p15),(p16)} The first therapeutic nanobody: caplacizumab (Sanofi), was approved by the FDA in February 2019 for the treatment of a rare blood clotting disorder, acquired thrombotic thrombocytopenic purpura (aTTP).^(p17) This approval has provided a significant boost for nanobody technologies, including their application to infectious diseases. Several studies have already reported the efficacy and safety of nanobodies targeting SARS-CoV-2 spike protein or its receptor binding domain (RBD).^{(p18),(p19),(p20),(p21),(p22)}

Despite their advantages, nanobody-based therapies face a fundamental limitation: nanobodies, like conventional antibodies, are primarily designed to recognize extracellular antigens and are not naturally internalized into cells.^(p23) This restricts their use in targeting intracellular components, including conserved viral replication machinery, such as RTCs. Unlike small-molecule drugs, which can freely diffuse across cell membranes or be actively transported into cells, nanobodies lack intrinsic mechanisms for cellular uptake, making their intracellular bioavailability a challenge, although some innovative approaches for intracellular proteins have been proposed.^(p24)

Overcoming this limitation requires novel delivery systems that can efficiently transport nanobodies into cells. Several strategies, including cell-penetrating peptides (CPPs) and chemical functionalization strategies,^{(p25),(p26)} induced transduction by osmocytosis^(p24) and transient charge-conversion^(p27) have been

explored to facilitate intracellular delivery of antibodies and nanobody therapeutics. The main obstacles of all the above technologies are insufficient control of delivery systems including their stability, appropriate ability for intracellular release and the number of active molecules delivered. Recent advances in mRNA-based therapeutics provide a promising approach by encoding intracellularly expressed nanobodies, bypassing the need for direct nanobody uptake.^(p8)

Targeting the RTC: from catalytic inhibition to protein–protein interference

In addition to the central RNA polymerase holoenzyme complex including RdRp (NSP12) and the NSP subunits NSP7 and NSP8 (two copies), coronavirus RTCs are composed of several essential cofactors required for RNA capping. These include the RNA-binding subunits NSP9 and NSP10, the helicase NSP13, the 3′/5′ exonuclease NSP14, the polyU-specific endoribonuclease NSP15 and the 2′-O-ribose methyltransferase NSP16. Given their importance for the functionality of the RTC complex, all these factors represent potential targets for drug design. Inhibition of the viral RNA replication leading to a block in viral propagation can be achieved using small molecules such as remdesivir, a prodrug of adenosine nucleotide analog GS-441524,^(p28) and molnupiravir, a prodrug of the ribonucleoside beta-D-N4-hydroxycytidine (NHC).^{(p29),(p30)} They are both prodrugs of nucleoside analogs that target, as their 5′-triphosphate metabolite, viral RdRp; and they have been recently approved as drugs to treat COVID-19 (Figure 1). Although adverse side effects have been reported, there is also a third approved antiviral drug: Paxlovid® (the combination of nirmatrelvir and ritonavir), which inhibits NSP5 – the main protease of SARS-CoV-2 (M^{Pro}) and, therefore, prevents the formation of the replication machinery from the viral polyprotein processing.^(p31)

We and others have also proposed small molecules targeting either protein–protein interactions (PPIs) within the RTC complex subunits (e.g., C459 targeting NSP10/NSP14 PPI)^(p32) or host cell enzymes hijacked by RTC subunits [e.g., AZ1 inhibiting the catalytic activity of the deubiquitinase ubiquitin-specific protease 25 (USP25)]^(p33) (Figure 1). In principle,

targeting the RTCs, independently of their specific enzymatic activities, could provide innovative therapies, which might synergize with the above catalytic inhibitors, and reduce the therapeutic impairment from resistant mutations that are hallmarks of monotherapies. To test this strategy, we have recently raised nanobodies targeting NSP9^(p34) – a protein acting as a catalytic substrate for the Nidoviral RdRp-associated nucleotidyltransferase (NiRAN)^(p35) activities of NSP12, which are directly linked to the initiation of viral RNA 5′ cap formation, an essential step for viral propagation. In addition to capping, NSP9 also has a direct role in the catalysis of the priming of viral RNA synthesis.^(p36) So, inactivating NSP9 is a potential new therapeutic strategy for the coronavirus family, and possibly the *Nidovirales* (Figure 1).

NSP9 oligomerization: a new avenue for broad-spectrum antivirals

Despite significant sequence differences (Figure 2a), invariance of the functionally crucial residues and the overall structural motif of NSP9 proteins are largely maintained within the *Nidovirales* order [i.e., coronaviruses whose genetic markers are the NiRAN catalytic domain in NSP12 (in addition to RdRp) and the NSP13 helicase]. During capping, NSP9 binds to the NiRAN domain and undergoes RNAlation through a covalent phosphoramidate bond at the N terminus with the nascent viral RNA, and subsequent NMPylation (nucleotide monophosphorylation) of the linked RNA 5′-pyrophosphate extremity. The NSP9 N-terminal sequence must contain an Asn2Glu3 fragment to match its interaction site in the NiRAN domain, as well as two Gly residues in position 100 and 104 in the C-terminal α -helix along with a few other invariances (residues 99, 102 and 103) to bind a nearby region of the NiRAN domain.^(p37)

The C-terminal α -helix of NSP9 is also crucial for the dimerization of the isolated protein and this overlap of two functions in the same area has often been confusing for the interpretation of the experimental data leading to the erroneous conclusion that 'during the infection NSP9 must form a dimer'.^(p38) This conclusion that was inferred from the inactivity of viruses expressing G100E or G104E NSP9 (i.e., two mutants unable to form a dimer in

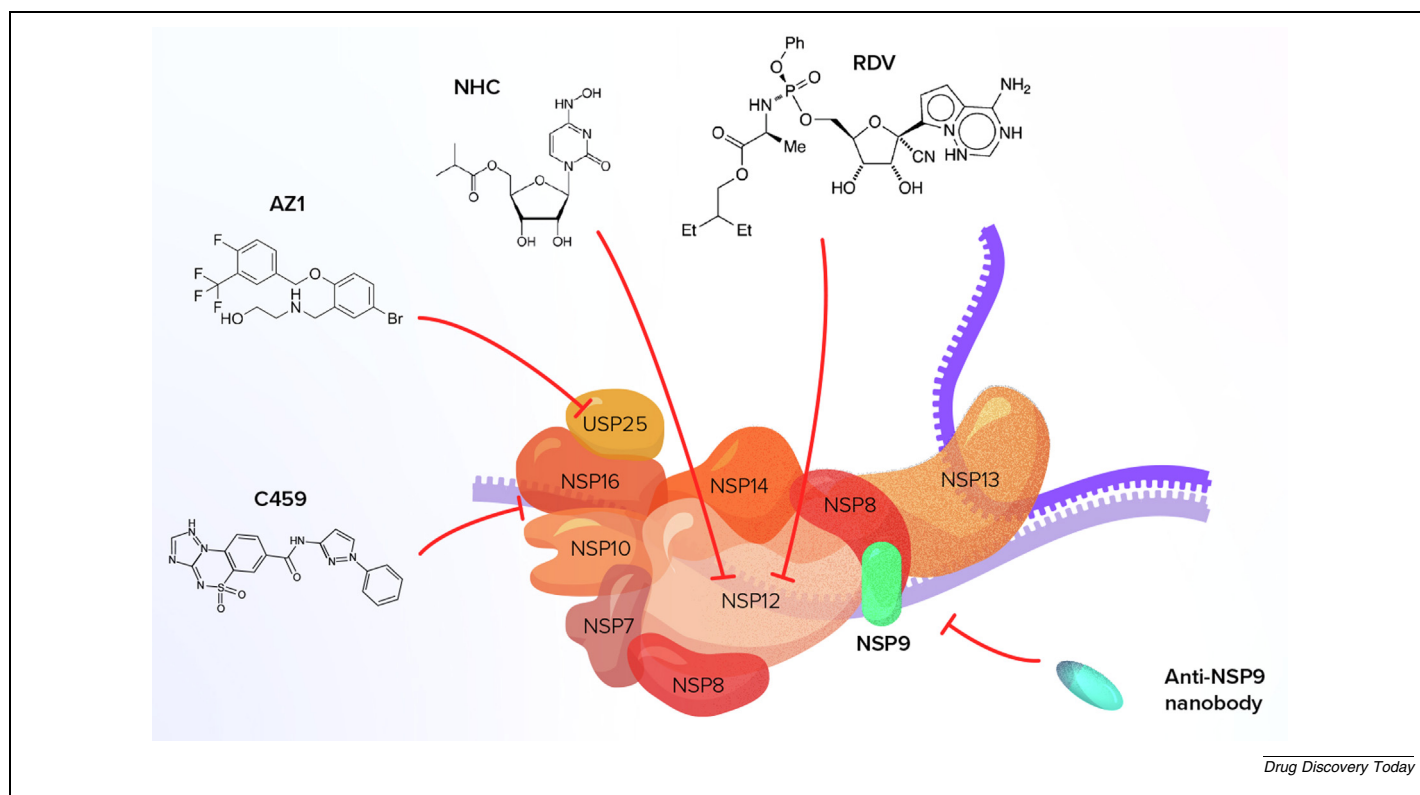


FIGURE 1

SARS-CoV2 RTC can be targeted by at least five molecules for efficient inhibition. Illustration of SARS-CoV-2 replication–transcription complex (RTC) and a few antiviral strategies: catalytic inhibitors remdesivir (RDV) and molnupiravir (beta-D-N4-hydroxycytidine, NHC); host target inhibitor anti-deubiquitinase ubiquitin-specific protease 25 (USP25), AZ1; RTC subunits NSP10/NSP14 protein–protein inhibitor, C459; and an anti-NSP9 nanobody molecule.

solution)^(p38) confirmed an analogous previous finding,^(p39) propagated until recently, and, paradoxically, affects even the RTC structural work.^(p37) Whereas only a single NSP9 molecule was observed in the complex, the authors concluded that “NSP9 may have an intermediate from a homodimeric state... to a monomeric state to be further assembled into RTC”. This conclusion was based on the lack of NMPylation at the NSP9 N-terminal residue, thereby suggesting a defective stoichiometry of the observed RTC assembly.

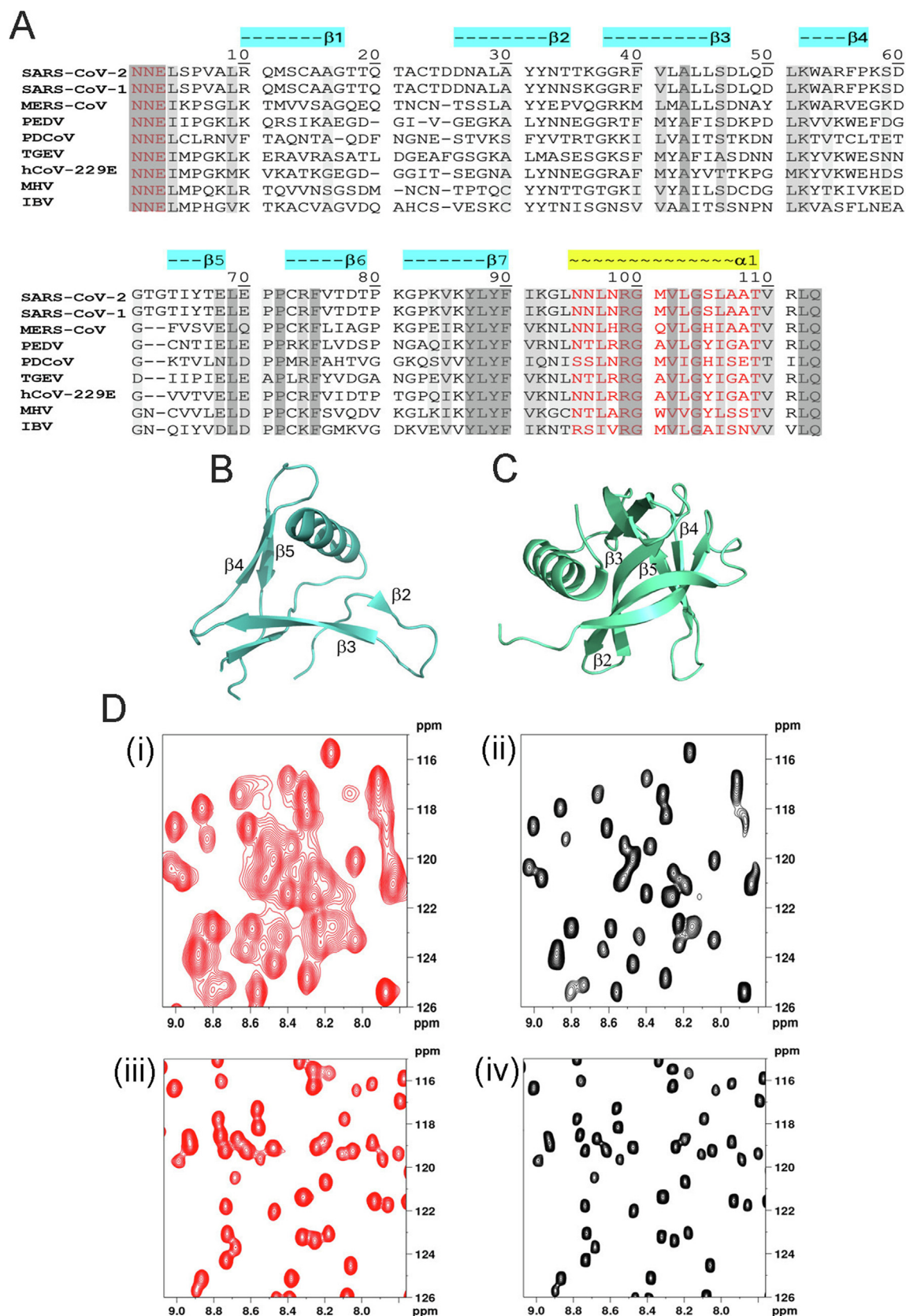
As subsequently pointed out by Wang et al. (2021),^(p40) the missing N-terminal NMPylation was the consequence of a non-native glycyl–seryl extension at NSP9 Asn1 surviving after the poly-His tag proteolytic cleavage. The NSP9 monomeric stoichiometry in RTC assembly was conclusively assessed by Baker and co-workers,^(p41) who also stressed the opportunity of targeting the NSP9 interaction with NSP12 to inhibit the viral replication using the recently reported NSP9-binding peptides derived from intrinsically disor-

dered protein regions of human proteome.^(p42) These peptides however do not suppress NMPylation and RNAylation of NSP9,^(p41) which suggests that their inhibition of the viral replication should be ascribed to the impairment of the other function of NSP9 (i.e., the priming of the viral RNA synthesis).^(p36)

By contrast, if NSP9 dimerization is not functional for the RNA capping – an essential process for viral proliferation – proposing dimerization-suppressing strategies to prevent viral infection^{(p38),(p43),(p44),(p45)} could prove self-defeating.^(p42) Instead, stabilizing NSP9 dimers or higher oligomers could provide a convenient solution to prevent the RTC assembly and the subsequent viral propagation.^{(p34),(p46)} Interestingly, the autophagic degradation of NSP9 aggregates has recently been involved in the inhibition of the replication of porcine epidemic diarrhea virus (PEDV).^(p47) The process requires the binding of NSP9 to Toll-interacting protein (TOLLIP), an autophagic cargo receptor that facilitates autophagic degradation

and therefore subtracts an essential RTC component. This relevance of NSP9 aggregation to exploit the natural autophagic mechanisms adds further confidence to antiviral strategies based on the stabilization of NSP9 oligomers.

To this end, we resorted to nanobodies raised against a more oligomerization-prone variant of NSP9 where the three cysteines in the sequence (positions 14, 23, 73) were replaced by serine residues.^(p34) The nanobodies raised in the presence of higher oligomers that significantly populate the association equilibria of the triple-serine NSP9 mutant, also recognized the wild-type protein whose oligomerization repertoire includes tetramers in addition to the prevalent dimers.^(p48) Consistently, the observed NMR evidence suggested the assembly of a NSP9 tetramer with four nanobody molecules, although the complex of a dimer with two nanobody units could not be ruled out.^{(p34),(p45)} The multimeric character of the NSP9 complex with our nanobodies was also confirmed by the crystal structure of a



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dimeric NSP9 assembly with two nanobody molecules^(p49) (Figure 2b, c).

The propensity to dimerization and higher association stoichiometries has long been observed in several NSP9 proteins.^{(p38),(p45),(p48),(p50),(p51)} Given the invariance of the functionally crucial residues and the overall conformational fold of NSP9 proteins, the strategic approach of stabilizing oligomers to prevent monomer recruitment in the RTC can be extended to the different NSP9 species from the coronaviruses of *Nidovirales* order (Figure 2a). A large number of determinations by size exclusion chromatography, dynamic light scattering, analytical ultracentrifugation and glutaraldehyde crosslinking indicate that NSP9 proteins also form higher oligomers in solution including trimers, tetramers and hexamers, albeit to sensibly reduced extents with respect to the predominant dimers often considered the only noteworthy species.^{(p38),(p39),(p45),(p50),(p51),(p52),(p53)}

A couple of recent studies^{(p41),(p54)} also stress the possibility of a naturally occurring NSP9 species [i.e., Middle East respiratory syndrome (MERS) coronavirus NSP9] that exists in solution only as a monomer based on a 12 kDa assessment of molecular mass by size exclusion chromatography. This conclusion might lead to overlooking the inherent propensity for oligomerization of the NSP9 motif that translates into complex association dynamics. As repeatedly shown by NMR,^{(p34),(p55),(p56)} the hallmark of any NSP9 motif pattern is the lack of signal from the N-terminal fragment(s) and part of the C-terminal α -helix region

(i.e., the dimerization interface) besides the attenuation (or loss) of several other signals from loop and β -strand regions [i.e., the higher oligomer interface(s)]. The signal cancellation is the direct consequence of the oligomerization dynamics which occurs in solution under conditions of intermediate exchange, on the chemical shift time scale, and broadens up to merging in the baseline, the limiting signals arising from the exchanging species. This feature is also observed in the NMR spectra of MERS coronavirus NSP9,^(p54) suggesting that the NSP9 oligomer stabilization strategy to prevent viral propagation can work even under conditions of substantial monomeric predominance. The oligomeric association propensity of NSP9 proteins can be readily appreciated from the pattern of their NMR spectra compared to proteins of similar size (Figure 2d).

In summary, growing evidence shows that the tendency of NSP9 to form oligomers – conserved across coronaviruses – is key to its function in viral replication. Stabilizing NSP9 oligomers can block its incorporation into the RTC, offering a promising antiviral strategy. Targeting NSP9 oligomerization, especially via nanobodies, could thus provide a broadly applicable means to hinder coronavirus replication. Given the therapeutic promise of targeting replication cofactors such as NSP9, it is crucial to consider the broader evidence supporting nanobodies as effective antiviral agents. Beyond our work on coronavirus replication cofactors, several studies have validated the antiviral potential of nanobodies against diverse viral tar-

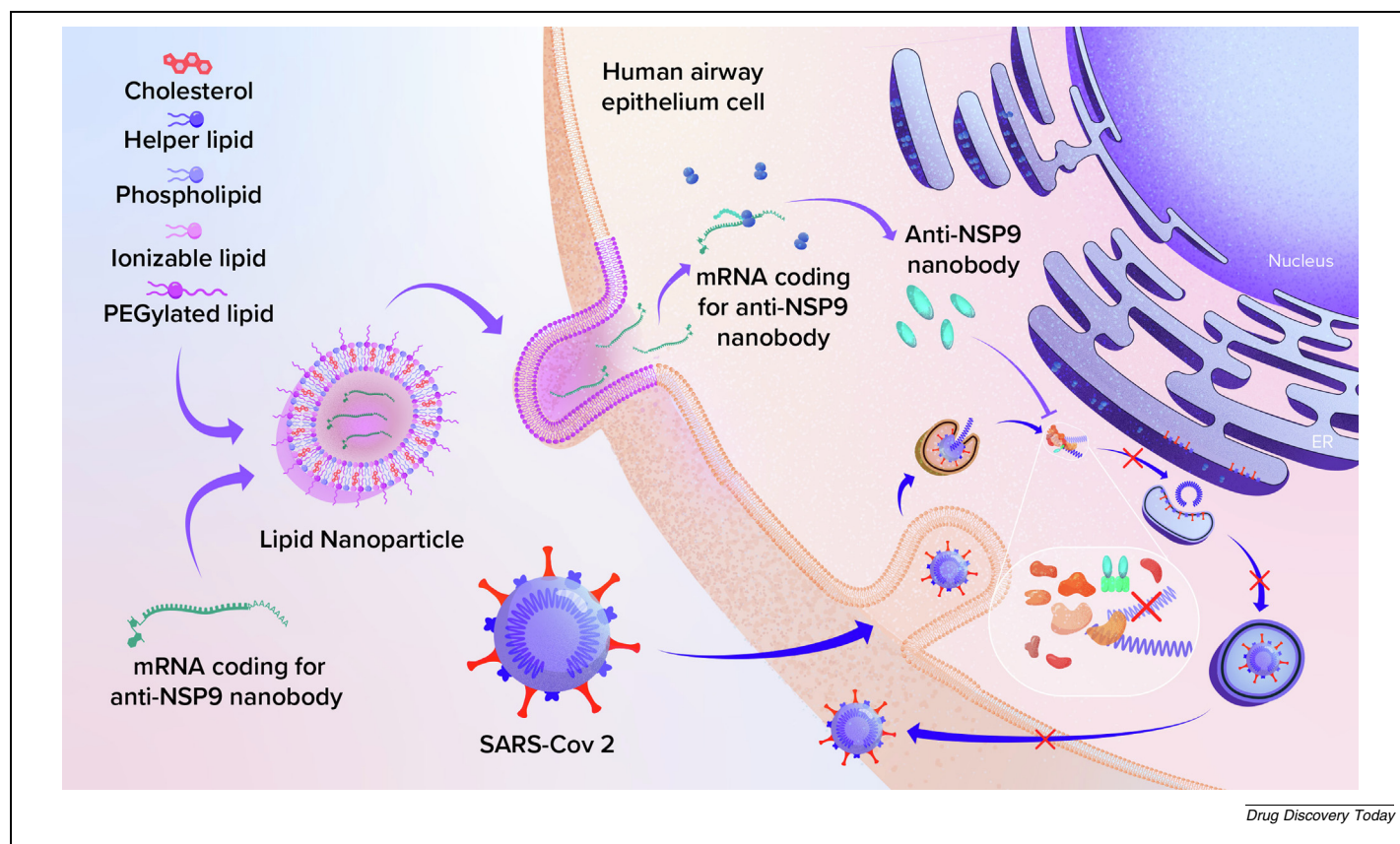
gets. For influenza A virus, nanobodies directed against the nucleoprotein or polymerase complex blocked replication in cell culture and protected mice from lethal infection, demonstrating direct intracellular antiviral activity.^(p57) In SARS-CoV-2, multivalent and hetero-bivalent nanobodies recognizing conserved epitopes of the spike receptor-binding domain potently neutralized all major variants, including Omicron.^{(p58),(p59)} Other reports described nanobodies targeting HIV-1 gp41/120 binding sites^{(p60),(p61)} that prevent viral entry or, in the case of Herpes simplex virus (HSV), where a bispecific nanobody binding to the gD prevents cell-to-cell transmission of HSV.^(p62) These studies collectively establish nanobodies as a potential versatile antiviral strategy for inhibiting key steps in viral lifecycles. Our approach extends this paradigm to intracellular RTCs by coupling nanobody specificity with efficient mRNA-based intracellular expression.

Nanobody-encoding mRNA molecules against viral RTCs

Nanobodies are mainly used to target cell-surface proteins for which targeted epitopes are available for easy binding of monoclonal antibodies or nanobodies. The mRNA-LNP technology that has already revolutionized the vaccine field is a viable tool to target intracellularly expressed viral proteins, such as NSP9. We recently demonstrated that an mRNA encoding a potent nanobody against NSP9, when encapsulated into LNPs, is delivered and translated in cell lines and

FIGURE 2

(a) Sequence alignment of the NSP9 proteins from severe acute respiratory syndrome coronaviruses (SARS-CoV-2, SARS-CoV-1), Middle East respiratory syndrome coronavirus (MERS-CoV), porcine epidemic diarrhea virus (PEDV), porcine delta coronavirus (PDCoV), transmissible gastroenteritis virus (TGEV), human coronavirus 229E (hCoV-229E), mouse hepatitis virus (MHV) and infectious bronchitis virus (IBV). The regions in red are involved in the contacts that SARS-CoV-2 NSP9 establishes with NSP12 N1RAN domain in the RTC complex.^(p37) Variable gray shadowing indicates different extents of sequence conservation. (b) The NSP9 monomer occurring in the crystal structure of the complex with 2NSP23 nanobody (PDB ID: 8dqu).^(p48) The C-terminal α -helix is oriented inwards between β 2– β 3 and β 4– β 5 sheets. Strand β 2 has a reduced extension in the crystal structure of the complex. (c) The NSP9 monomer in the canonical dimer structure (PDB ID: 6wxd) where the C-terminal α -helix is outside the compact β -sheet region. (d) ^{15}N - ^1H HSQC region of SARS-CoV-2 NSP9 (131 μM) processed (i) without and (ii) with resolution enhancement (squared sine shifted by $\pi/2$ and $\pi/4$, respectively), to highlight the broadening undergone by the cross-peaks of a 13.4 kDa protein because of oligomerization equilibria. This can be appreciated by inspection of the analogous ^{15}N - ^1H HSQC region of a human lysozyme (14.9 kDa) sample (50 μM) that is shown in subpanels iii and iv, after using the same processing as in i and ii, respectively. The spectra of both proteins were collected at 298 K with the same spectrometer (14 T, i.e., 600 MHz ^1H , 60.8 MHz ^{15}N) and acquisition conditions. The width increase of NSP9 cross-peaks reflects the different tumbling rates of the various oligomers and their exchange process occurring at faster rates with respect to their limiting chemical shift difference (Δ). Locally, where the limiting Δ values match the exchange rate, the specific cross-peaks broaden up to merging in the baseline and becoming unobservable (intermediate exchange limit). For SARS-CoV-2 NSP9 this signal loss is observed for many residues in the C-terminal α -helix and N-terminal region involved in the dimerization interface, whereas substantial attenuations are also present in β -strand and loop regions^{(p34),(p54),(p55)} involved in higher oligomer interface. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**FIGURE 3**

Inhibition of SARS-CoV2 replication using an mRNA coding for anti-NSP9 nanobodies. The mRNA encoding an anti-NSP9 nanobody is encapsulated into LNPs. The nanobody mRNA–LNP is up taken by cells, the mRNA is liberated and then translated. Upon viral infection, the nanobody, produced intracellularly, targets NSP9 and inhibits RTC function.

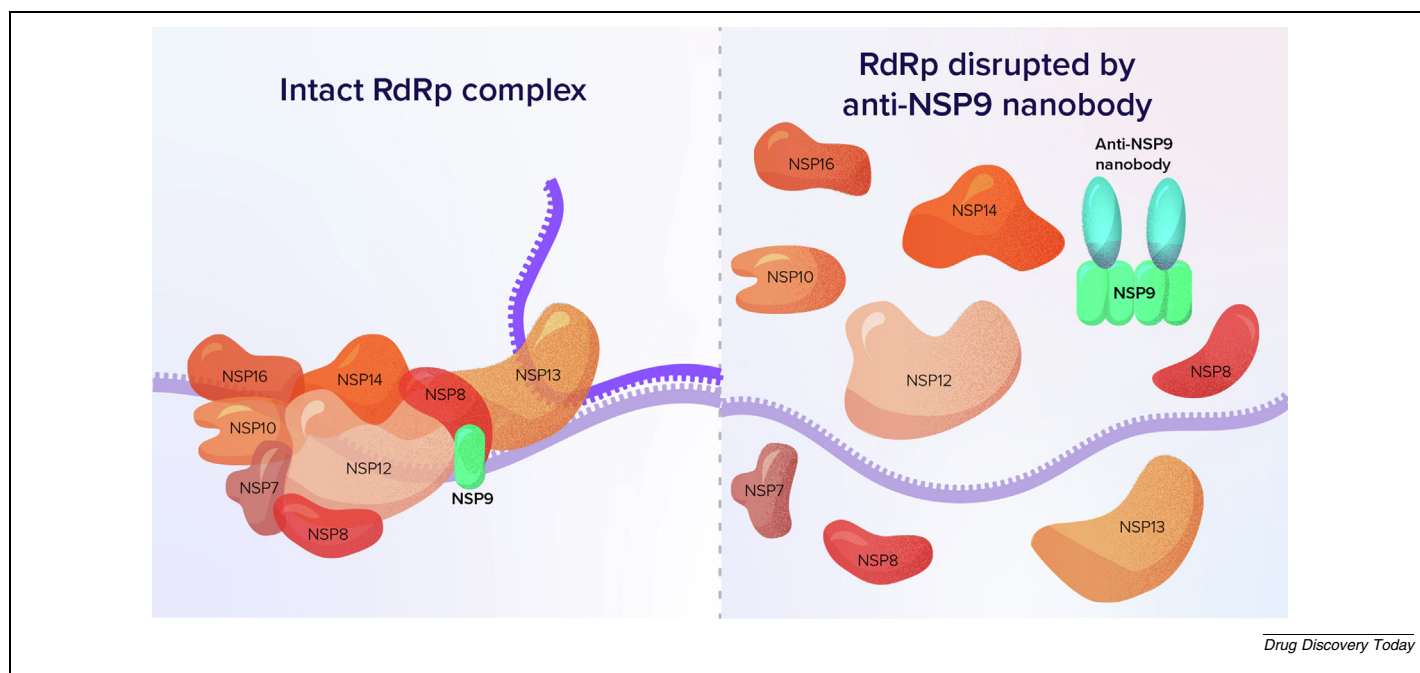
human tissues (Figure 3). Once intracellularly expressed, this nanobody interacts with NSP9 and inhibits replication of multiple variants of SARS-CoV2.^(p8) Therefore, this specific nanobody mRNA–LNP formulation has strong antiviral potential.

This example illustrates the feasibility of intracellular nanobody expression, a concept supported by the rapid evolution of mRNA–LNP technology. The success of mRNA vaccines against COVID-19 has established LNP-mediated mRNA delivery as a clinically validated platform for *in vivo* protein expression. Synthetic mRNAs incorporate optimized cap analogs, untranslated regions and modified nucleosides (e.g., N1-methylpseudouridine) to enhance stability and reduce innate immune activation.^{(p63),(p64)} When encapsulated in ionizable LNPs, these molecules enter host cells via endocytosis and release the mRNA into the cytosol for translation by ribosomes. This

strategy circumvents the poor intracellular access of antibody biologics by enabling direct synthesis of therapeutic nanobodies within infected cells. The transient and tunable expression of mRNA-encoded nanobodies provides a flexible and rapidly deployable modality for antiviral intervention across multiple virus families.^{(p65),(p66)}

Using primarily NMR and *in silico* modeling, the inhibitory activity of the anti-NSP9 nanobody is mechanistically explained by a tight and specific binding to the NSP9 protein that stabilizes non-functional NSP9 tetramers and, perhaps, oligomers of lower and higher molecular weight.^{(p34),(p46)} As already described, this results in depleting the pool of functional monomeric NSP9 required to promote assembly of a functional RTC complex and, thus, its binding to the viral RNA (Figure 4). We demonstrated that this mechanism inhibits replication of several SARS-CoV-2 variants in cells and human airway

epithelia infected with the virus. So, anti-NSP9 nanobodies have potential to work as strong antivirals that inhibit viral replication by repressing the formation of functional RTC complexes.^(p8) The development of vaccines based on mRNA–LNP technology enabled control of the COVID-19 pandemic mainly owing to prior knowledge that SARS-CoV spike protein is the main surface-exposed protein of coronaviruses, exploiting rapid translation of exogenous mRNA while using pseudo-nucleotides that avoid immediate destruction by the host innate immunity. Translating the mRNA–LNP technology in the design of biologic antivirals is compelling because it would offer a unique opportunity of targeting the most conserved mechanisms of viral replication, as demonstrated in our study targeting NSP9 of *Nidovirales*.^(p8) Besides the human acute respiratory syndromes, the coronaviruses belonging to that order

**FIGURE 4**

Proposed mechanism underlying the disruption of the SARS-CoV-2 RTC complex by anti-NSP9 nanobody. Once expressed intracellularly, the anti-NSP9 nanobody interacts with NSP9 and stabilizes a non-functional tetrameric form of the protein. We propose that under these conditions the RTC complex does not assemble and this leads to inhibition of viral replication.

are responsible for many serious diseases affecting other animals, with relevant economic consequences when considering livestock. The coronavirus NSP9 sequences of Figure 2a present a minimum 40–50% (avian infectious bronchitis virus) of sequence similarity with SARS-CoV-2 NSP9 and conserve all the main features that determine the behavior of the protein, suggesting that the strategy of targeting NSP9 to stabilize its oligomerization with nanobodies administered by mRNA-LNP technology could also be extended to veterinary contexts. In addition to possible benefits to swine production through control of porcine epidemic diarrhea (PEDV), delta coronavirus (PDCoV) and transmissible gastroenteritis (TGEV),^(p67) further advantages might ensue for pets by targeting feline and canine coronaviruses (FCoV, CCoV)^{(p68),(p69)} to stop outbreaks of feline infectious peritonitis and canine enteritis, as well as for cattle breeding to defeat respiratory illness in adult cattle and dysentery in calves by targeting bovine coronavirus (BCoV).^(p70) All of these viruses belong to *Nidovirales* and

direct sequence retrieval from available databanks, for example GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>), confirms high levels of sequence similarity (50–75%) between the NSP9 sequence from SARS-CoV-2 and those from FCoV, CCoV and BCoV.

Concluding remarks

The current antiviral arsenal remains limited, with only ~90 molecules available today, and with a strong bias toward viral enzyme inhibitors.^(p71) To better prepare for the next pandemics, we propose a systematic virus-family-based broad-spectrum antivirals strategy that would combine experimental and AI-driven small molecules targeting viral enzymes and complex assembly (virus–virus and virus–host contactomes), and innovative biologics based on highly potent protein-based antivirals such as nanobodies. We have demonstrated that this strategy could enable expanding antiviral drugs by showing that the RTC of coronaviruses can be targeted by at least five different molecules: four small molecules including cat-

alytic inhibitors remdesivir (RDV)^(p28) and molnupiravir (NHC),^{(p29),(p30)} AZ1 targeting interaction between NSP16 and the host protein USP25^(p33) or C459 targeting interaction between NSP10 and NSP16^(p32) and a nanobody (anti-NSP9) expressed via mRNA-LNP technology^(p8) (Figure 3). The non-structural components of the RTC complex do not show variability and are conserved across coronaviruses. Therefore, by targeting intracellular viral proteins, it is possible to tackle one of the major hurdles faced when designing antivirals against surface proteins, their high mutagenic tendency, which led to highly inefficient drugs. This experimental pipeline has, therefore, potential implications for the design of highly specific drugs to tackle viral pandemics but also other diseases, such as metabolic ones, that rely on the overexpression of intracellular factors. In addition, just as HIV is contained by clinical use of at least a combination of three different therapies from an arsenal of ~40 small molecules, synergizing in their ability to inhibit viral replication and control of inevitable resistant mutations, current

technology advances should allow the research community to propose a cocktail of antivirals for any family of priority pathogens.

Conflicts of interest

P.P. and G.E. are part of a US patent filed by New York University in Abu Dhabi.

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fellow of Les Amis des Instituts Pasteur à Bruxelles.

Author contributions

P.P. and **J-C.T.** conceived the perspective idea and wrote the manuscript together with **G.E.** and **J.B.**

Data availability

No data was used for the research described in the article.

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