



Current concepts in the development of therapeutics against human and animal coronavirus diseases by targeting NP



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ABSTRACT

The coronavirus (CoV) infects a broad range of hosts including humans as well as a variety of animals. It has gained overwhelming concerns since the emergence of deadly human coronaviruses (HCoVs), severe acute respiratory syndrome coronavirus (SARS-CoV) in 2003, followed by Middle East respiratory syndrome coronavirus (MERS-CoV) in 2015. Very recently, special attention has been paid to the novel coronavirus disease 2019 (COVID-19) caused by SARS-CoV-2 due to its high mobility and mortality. As the COVID-19 pandemic continues, despite vast research efforts, the effective pharmaceutical interventions are still not available for clinical uses. Both expanded knowledge on structure insights and the essential function of viral nucleocapsid (N) protein are key basis for the development of novel, and potentially, a broad-spectrum inhibitor against coronavirus diseases. This review aimed to delineate the current research from the perspective of biochemical and structural study in cell-based assays as well as virtual screen approaches to identify N protein antagonists targeting not only HCoVs but also animal CoVs.

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Abbreviations: CoV, coronavirus; HCoVs, human coronaviruses; SARS-CoV, severe acute respiratory syndrome coronavirus; MERS-CoV, Middle East respiratory syndrome coronavirus; COVID-19, coronavirus disease 2019; SP, spike protein; RNP, ribonucleoproteins; MP, membrane protein; nsp3, the nonstructural protein 3; E, envelope protein; IFN, interferon; NTD, N-terminus RNA-binding domain; CTD, C-terminus dimerization domain; RBD, RNA-binding domain; MHV, mouse hepatitis virus; IBV, infectious bronchitis virus; AMP, UMP, GMP and CMP, ribonucleoside 5'-monophosphates; HIV, human immunodeficiency virus; FIPV, feline infectious peritonitis virus; CCoV, canine coronavirus; BCoV, bovine coronavirus; ECoV, equine coronavirus; TCoV, turkey coronavirus; PEDV, Porcine epidemic diarrhea virus; TGEV, transmissible gastroenteritis virus; PDCoV, porcine deltacoronavirus; PRCV, porcine respiratory coronavirus; SeCoV, swine enteric coronavirus; shRNAs, short hairpin RNAs; FECV, feline enteric coronavirus; siRNA, small interfering RNA.

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1. Introduction

Human coronaviruses (HCoVs), including HCoV-229E, HCoV-NL63, HCoV-OC43 and HCoV-HKU1 typically cause subclinical infections [1]. However, three novel coronaviruses (CoVs) with high mortality rates have emerged since 2003 and possess severe threats to humans. CoVs have drawn great concern since the outbreak of severe acute respiratory syndrome (SARS) that led to approximately 800 deaths with a fatality rate of approximately 40% in aged people [2]. Ten years later, another CoV, Middle East respiratory syndrome (MERS), had 30% mortality rate and caused more than 2,000 cases of infection worldwide [3].

However, neither specific therapeutic agents nor approved vaccines are approved for the treatment or prevention of CoV infections. In January 2020, an outbreak of COVID-19 caused by a novel CoV, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was documented in China [4]. This newly emerging coronavirus has rapidly spread worldwide and been declared a pandemic. Up to January 2021, over 96 million people have been infected by SARS-CoV-2 and the death toll has reached 2 million cases.

Several vaccine candidates are currently undergoing clinical trials, with results indicating that adenovirus-based and mRNA-based SARS-CoV-2 vaccines are clinically effective, as both can induce significant humeral immune responses with minimal side effects [5–7]. Despite promising progress in vaccine development, total cases of hospitalization and death are still increasing, making development of therapeutics an urgent matter. As the interface between spike protein of CoVs and its host receptor is essential for virus entry, efforts have been notably made in finding molecules against the function of CoV spike [8,9]. For instance, two research groups have developed *in silico* platform to screen FDA-approved small molecule libraries and natural compounds that can target the interface between spike and host receptor [10,11]. However, accumulated evidence indicated SARS-CoV spike protein (SP) had a higher mutation rate and less stable than nucleocapsid protein (NP) [12,13]. Hence, although most studies have focused on the SP of CoVs, a growing amount of evidence showed that the NP is a potential target for drug development.

The NP structures of many CoVs, including those highly fatal emerging CoVs, reveal similarities in the structure and function of the N- and C- termini, which are responsible for RNA binding and oligomerization, respectively. Identifying such residues yields an opportunity to design novel inhibitors or screen for potentially effective molecules in current drug libraries. Due to these unique features, the NP is a promising target for development of broad-spectrum anti-coronavirus therapeutics [14].

In this mini-review, we emphasized the structure–function analysis of the CoVs' NPs and summarized current findings on structure-based development of therapeutics for both human and animal CoVs by targeting NPs.

2. The function of NPs in coronavirus

The NP is a versatile protein which has various bio-functions, including oligomerizing NPs, packing viral genome RNA into ribonucleoproteins (RNP) and interacting with other viral proteins. For instance, the NP associates with the membrane (MP) and the nonstructural protein 3 (nsp3) [15]. Throughout viral replication, NP-MP interaction contributes to viral core formation, assembly, budding, and envelope formation. An ionic interaction between the C-terminal region (composed of the residues 237–252) of the MP and the NP leads to genome encapsidation of budding viral particles [16–18]. Moreover, assembly of coronavirus virions requires dimerization of NPs [19–21] and association with viral genomic

RNA that ultimately forms RNPs [16,22–26]. In addition to RNP formation, interactions among the four structural proteins (NP, MP, E, and SP) and acquirement of viral envelopes from the host membrane at budding sites are also critical for virion formation and infectivity. Although NPs are not required for the virion envelope formation [27–29], overexpression of NP significantly increased virus production [30].

In addition to the regulatory role in viral RNP assembly and genome budding, CoV NPs facilitate viral propagation by modulation of cellular machinery. For instance, the SARS-CoV NP could interrupt the host cell cycle via inhibition of cyclin-CDK activity, which leads to the arrest of S phase progression [31]. Furthermore, CoVs counteract cellular innate immunity, particularly regulation of interferon (IFN) production, to facilitate viral infection. It is noted that the SARS-CoV NP is one of the effectors of this mechanism, antagonizing IFN- β production through the inhibitory effect on IRF-3 and NF- κ B activation [32].

3. Structure and function analysis of NPs

NPs are abundant structural proteins in CoVs. The primary function of NPs is to bind the viral RNA genome, form the RNP, and further compress it into a compact virion core. Previous studies [12,13] and as illustrated in Fig. 1, sequences and structures of NPs are relatively conserved among CoVs. Two functional domains were found in the NPs of CoVs, the N-terminus RNA-binding domain (NTD) and the C-terminus dimerization domain (CTD), which are connected by a central Ser/Arg-rich flexible linker. The NTD and CTD of NPs are responsible for association with viral RNA and formation of NP oligomers, respectively [20,33]. The central linker region, with its multiple phosphorylation sites [34], has also been demonstrated to be essential in RNA-binding [35]. Initially, the CTD was the first domain found to perform oligomerization; more recent evidence has indicated that NTDs also form homodimers, further leading to NP oligomerization via protein–protein interactions [36].

The RNA-binding domain (RBD) has been mapped to the NPs. The minimal region responsible for RNA-binding activity was located from residue 177 to 231 in mouse hepatitis virus (MHV) [22]. Of note, the NP NTD of various CoVs shares conserved secondary structures, i.e. right-handed (loops)-(β -sheet core)-(loops) sandwiched folds [14], which possess a key scaffold for RNA binding. Generally speaking, an NTD is composed of a five-stranded antiparallel β sheet structure with the topology β_4 - β_2 - β_3 - β_1 - β_5 [37]. Several positively charged amino acids – for instance Arg 106, Arg 107, and Arg 117 residues – located at the protruding loop of the NTD in HCoV-OC43, are important for electrostatic interactions with the phosphate backbone of RNA [38]. Moreover, two tyrosine residues in the β_5 region of NTDs are conserved in both human and animal CoVs (Fig. 1A, grey arrows), which were proposed to be essential for ribonucleotide binding [14] and correlated with viral replication. Substitution of Tyr 127 and Tyr 129 with alanine resulted in attenuation of MHV replication [39]. Alanine substitution of Tyr 94 at the NTD of infectious bronchitis virus (IBV), corresponding to Tyr 126 of HCoV-OC43 and Tyr 129 of MHV, has consistently led to significantly reduced RNA-binding activity and consequently, decreased infectivity has been demonstrated in Vero cells [40]. In order to understand molecular interaction between NTDs and RNA, the crystal structure of the NTD of HCoV-OC43 in a complex with ribonucleoside 5'-monophosphates (AMP, UMP, GMP and CMP), has been resolved by means of X-ray diffraction [36]. Residues including Ser 64, Gly 68, Arg 122, Tyr 124, Tyr 126, and Arg 164 are located in the center of the RNA-binding pocket in HCoV-OC43 NTD, a composition conserved in other CoV viruses (Fig. 1B). Lin and colleagues, further

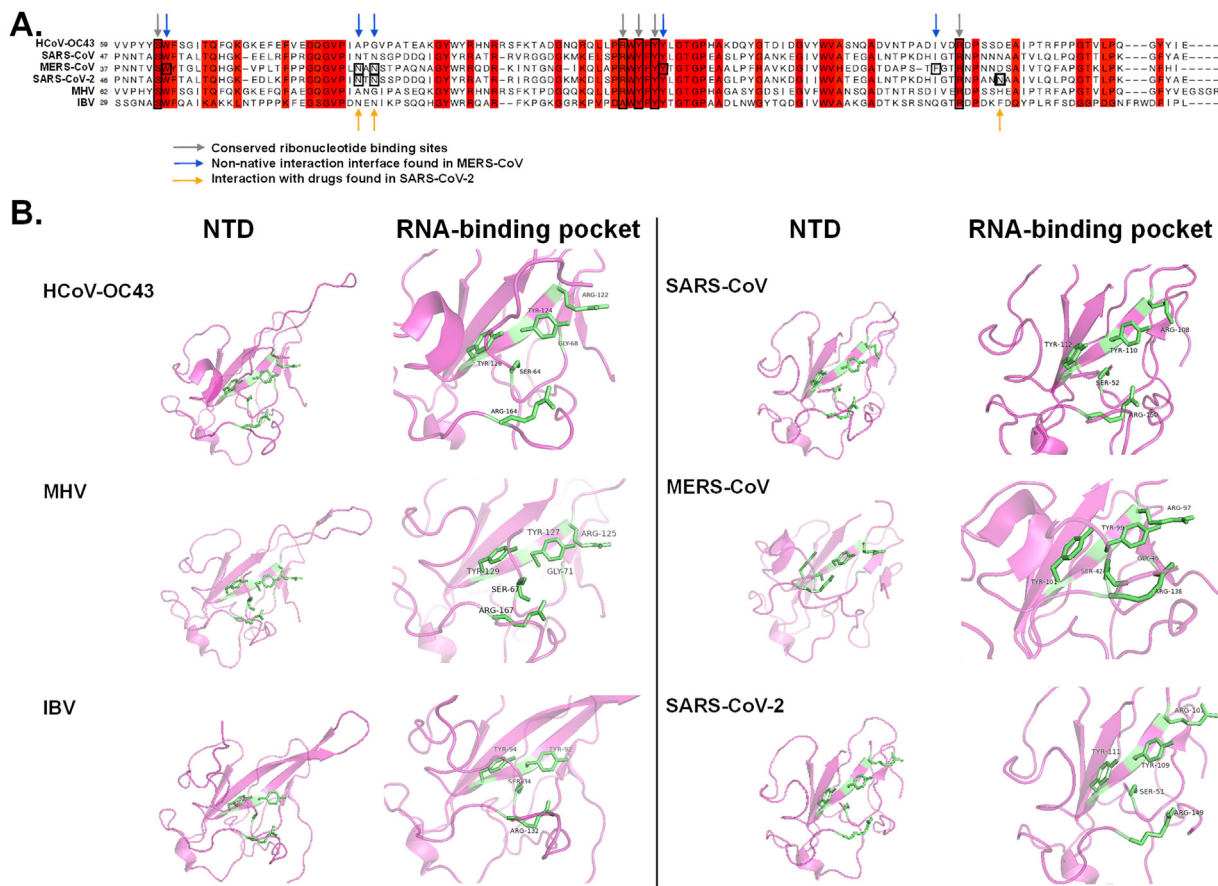


Fig. 1. Sequence alignment and structure analyses of NTD of CoV NP. (A) Multiple sequence alignment of HCoV-OC43 (NC005147), SARS-CoV (NC004718), MERS-CoV (NC019843), SARS-CoV-2 (NC045512), MHV (NC001846) and IBV (AY692454). The highly conserved residues were highlighted in red. Grey arrows indicate conserved RNA binding sites identified in previous reports [39,48,51,61]. Blue arrows indicate important residues for non-native oligomerization [48]. Yellow arrows indicate binding sites between potential anti-SARS-CoV-2 compounds and N protein [51]. (B) NTD and RNA binding pockets of HCoV-OC43 (PDB: 4J3K), MHV (PDB: 3HD4), IBV (PDB: 2GEC), SARS-CoV (PDB: 2OG3), MERS-CoV (PDB: 6KL2) and SARS-CoV-2 (PDB: 6WKP) N protein. Green sticks indicate RNA-interaction residues. HCoV-OC43, human coronavirus OC43; MERS-CoV, Middle East respiratory syndrome coronavirus; SARS-CoV, severe acute respiratory syndrome coronavirus; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; MHV, mouse hepatitis virus; IBV, infectious bronchitis virus. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

showed that the interaction between an NP and ribonucleoside 5'-monophosphate mainly relies on hydrogen (H) bonds, ionic bonds, and π - π stacking [36]. The depiction of the crystal structure of HCoV-OC43 NP-NTD complexed with AMP identified a distinct ribonucleotide-binding pocket composed of Arg 122, Tyr 124, Tyr 126, and Arg 164. Tyr 124, located on the surface of the NP, directly interacted with the AMP base through π - π stacking. Moreover, H-bonds were formed between the phenolic hydroxyl group of Tyr 126 and the adenine ring of AMP. This implies that Tyr124 and Tyr126 are essential for recognition and interaction with RNA. Since the two residues are sequentially and structurally conserved in NPs among other CoVs, they could be a potential cross-strain target for drug development.

In addition to the one on the N-terminus, a second RBD has been mapped to the C-terminal region of the NP, between residues 363 to 382 [41]. In addition to RNA-binding, CTD also contributes to the formation of oligomers. As revealed by native gel electrophoresis and size-exclusion chromatography, in the absence of viral genomic RNA, NPs of SARS-CoV form dimers at low concentrations and high-order oligomers at higher concentrations [42]. It has been demonstrated that oligomerization of NPs maintains intact function and is regarded as a crucial step for coronavirus genome assembly [43]. Based on previous reports, the formation of high order NP oligomers is possibly due to self-association of CTDs (residues 283–422) in a concentration-dependent manner [41].

4. Inhibitors of NPs

The emergence of high mortality SARS-CoV in 2003 increased the urgency to develop effective drugs against human CoVs. As the NP is an essential viral protein with multiple functions and shares a relatively high level of sequence similarity with other members of *Coronaviridae*, it is a suitable target for drug development.

Many of proteins exert their bio-functions by formation of protein: protein interactions (PPI). The interfaces of native PPI are specific and flexible that serve as promising scaffolds for drug design. Of note, interactions of the genomic RNA of CoVs occur through the NTDs of NPs [36], and owing to the conserved native of NP structure, attempts were made to identify small molecules that could bind and further modulate its physiological function [44]. Several signature residues involved in the intermolecular interaction of NPs have been depicted. A ribonucleotide-binding pocket with conserved residues (Arg 122, Tyr 124, Tyr 126, and Arg 164) has been found among different human and animal CoVs (as indicated with grey arrows in Fig. 1A). Based on this structural feature, Chang et al., identified a lead compound (H3) that could inhibit the RNA-binding activity of HCoV-OC43 NPs [44]. As revealed in the structure prediction of HCoV-OC43 NP-NTD in a complex with 2-morpholin-4-yl-ethylamino, Tyr124 directly interacted with quinolone group, and residue Arg164 bonded with the

quinoline-5,8-dione ring of 2-morpholin-4-yl-ethylamino with hydrogen bonds, which form a specific binding area with Phe57 and Arg171. Previously, another HCoV-OC43 NP inhibitor, PJ34, has been shown to interact with Phe66 and Tyr124 of NPs through π - π stacking, albeit with a lesser inhibitory effect [43]. Structural superimposition of the HCoV-OC43 NP-NTD with H3 and PJ34 revealed that the branched moiety of PJ34 and morpholin moiety of H3 docked in opposite directions on the HCoV-OC43 NP. Since interaction of H3 molecule highly resembles that of nucleotide AMP, the difference in docking orientation of PJ34 moiety could partially explain its decreased inhibitory effect relative to H3.

Changhyun Roh and colleagues developed a platform for screening inhibitors of the SARS-CoV NP. Candidate compounds were selected based on their ability to compete with quantum dots-conjugated specific RNA oligonucleotides at the RNA-binding site of NP. (–)-catechin gallate and (–)-gallicocatechin gallate showed high binding ability with the RBD of SARS-CoV NPs [45]. Both compounds are phytochemicals with antioxidant, anti-inflammatory, and antiviral activities; the former has been reported to inhibit human immunodeficiency virus (HIV) infection via binding with CD4, the receptor for HIV, which ultimately blocks virus entry [46,47]. As analysis on an RNA oligonucleotide biochip platform, (–)-catechin gallate and (–)-gallicocatechin gallate were able to interrupt the binding between NP and RNA; the concentration required for 50% inhibition (IC_{50} value) was approximately 0.05 μ g/ml. Nevertheless, the anti-SARS-CoV activity of these compounds against genuine virus has not yet been investigated *in vitro* or in animal models.

More recently, instead of stabilization of native PPI, an alternative strategy for anti-viral drug discovery was established to identify small molecules that could stabilize the non-native PPI interface of NP [48]. Lin et al., revealed that 5-benzyloxygr-amine, in the Acros and ZINC drug databases, can bind and stabilize non-native interfaces between MERS-CoV NP oligomers, ultimately jeopardizing biochemical activity of the target protein [48]. Specific stabilization mediated by 5-benzyloxygr-amine led to abnormal aggregation of the full-length MERS-CoV NPs, inhibiting MERS-CoV replication in Vero E6 cells with EC_{50} of 32.1 μ M. The underlying mechanism was further investigated through structure-based study. It was proposed that Trp43, Asn66, Asn68, Tyr102, and Phe135 on the flexible region of NTDs form a conserved hydrophobic pocket (Fig. 1A, blue arrows), which accommodates the side chain of Met38 of the other NP monomer via hydrophobic contact. The Trp43 residue is important in maintaining the hydrophobic pocket for interaction with the vector-fusion residues of His37 and Met38 from the second monomer of MERS-CoV NP. It was demonstrated that 5-benzyloxygr-amine was able to replace the vector-fusion residues, and that in turn stabilized the NTD of MERS-CoV NPs. Although developing compounds to stabilize the non-native interfaces between proteins is not a conventional means for screening therapeutic compounds, it has been used to identify new anti-viral lead compounds. Two such molecules, nucleozin and S119, have been found to alter the oligomerization state of influenza virus NPs. Abnormal NP aggregates were formed and antagonized the original nuclear localization [49]. As the relatively high similarity of influenza NPs has been documented, the broad-spectrum antiviral effects of these drugs were demonstrated in cell cultures and mouse models.

Structural analysis indicated that the residues (Asn48, Asn49, Thr50, and Ala51) in the N terminal tail of NPs in HCoV-OC43 are more flexible and possibly serve as the binding pocket for viral RNA genomic high order structure [14]. By *in-silico* screening 56,079 compounds, Sarma and colleagues identified two compounds (ZINC00003118440 and ZINC0000146942, the theophylline and pyrimidone derivatives, respectively) that effectively interact with the NTD of the NPs (i.e. PDB ID: 2OFZ) of SARS-

CoV-2 [50]. More specifically, by means of molecular modeling and docking, Yadav *et al.*, conducted a high throughput virtual screening against the RNA-binding potential of NPs (PDB ID: 6YVO) [51]. First, five active sites responsible for RNA-binding were identified, of which three with the highest scores were chosen for docking against the selected ligands: 8722 antiviral drugs from Asinex databases and 265 FDA-approved drugs (from PubChem) for infectious diseases. Ultimately, two antiviral moieties (5817 and 6799) and one FDA drug molecule (Zidovudine) demonstrated great affinity to the three active sites. As evidenced by molecular dynamics simulations, each drug molecule forms 4 to 5 significant hydrogen bond interactions with NPs. Interestingly, most hydrogen bond interactions of the drugs are associated with Asn residues of the NP (Asn75, Asn77, and Asn154 residues, as indicated in yellow arrows in Fig. 1A). Moreover, results from this screening also indicated that many FDA-approved molecules (e.g. anti-HIV drugs and anti-herpes drugs) are active in interactions with the NP. Hence, for urgent use, drug repurposing is cost-effective and crucial for delivering new treatments against COVID-19. The inhibitors against human CoVs described in this review was concisely summarized in Table 1.

5. Therapeutics against NPs of domestic animal CoVs

CoVs also infect farm animals and companion animals, causing huge economic impact and loss of nonhuman companions. The total range of pathogenicity within the broad coronavirus family encompasses but is not limited to gastroenteritis in pigs, bronchitis in chickens, and peritonitis in felines. Phylogenetic analysis of NPs within the family of CoVs shows four major groups: feline infectious peritonitis virus (FIPV), canine coronavirus (CCoV) and two porcine coronaviruses in group I; bovine coronavirus (BCoV), equine coronavirus (ECoV), and MHV in group II; turkey coronavirus (TCoV), IBV and marine coronaviruses in group III; and SARS-CoV, MERS-CoV and SARS-CoV-2 in a distinct group IV (Fig. 2). The following discusses current preclinical small molecules, such as drugs, antibodies, and RNA interference techniques against the NPs of domestic animal CoVs.

5.1. Porcine coronaviruses

Porcine coronaviruses can be classified into the genera *Alpha-coronavirus*, *Betacoronavirus* and *Deltacoronavirus* in the family *Coronaviridae*. Porcine epidemic diarrhea virus (PEDV) and transmissible gastroenteritis virus (TGEV), belonging to the *Alphacoronavirus* genus, are two major pathogens that cause gastroenteritis in pigs, with especially severe cases in neonatal piglets [52]. An emerging porcine deltacoronavirus (PDCoV) reported in 2009 has pathological lesions that are clinically indistinguishable from those of PEDV and TGEV. The high evolution rate of CoVs has led to many variants – a non-pathogenic porcine respiratory coronavirus (PRCV) descended from TGEV has specific mutations in its spike gene. Co-circulation of porcine CoVs has been described worldwide, a circumstance that eventually generated a novel pathogenic recombinant variant containing PEDV SP on a TGEV backbone (swine enteric coronavirus, SeCoV) [53]. To date, there are neither specific treatments nor antivirals available for porcine coronaviruses, making it difficult to control the rapid emergence and spread of these viruses.

The classical PEDV has caused epidemics in Europe since the 1970s, and the outbreaks in China in 2010s were caused by the emergence of highly virulent PEDV strains. Commercial PEDV vaccines derived from classical PEDV are not effective against highly virulent strains. Deejai *et al.*, developed a virtual screening system based on the three-dimensional structure of PEDV NPs. The results

Table 1
Therapeutics against NPs of human CoVs.

Compounds	Virus	Binding sites	Mechanism	Assay system or simulation modelling	Dose	Reference
H3, 6-chloro-7-(2-morpholin-4-yl-ethylamino) quinoxaline-5,8-dione	HCoV-OC43	Phe66, Tyr124, Arg164, Phe57, Ala171	Decrease of RNA-binding capacity of NP	<i>In vitro</i> assay, surface plasmon resonance (SPR)	2 mM	[44]
PJ34, N-(6-oxo-5,6-dihydrophenanthridin-2-yl)(N,N-dimethylamino)acetamide hydrochloride	HCoV-OC43	Ser 64, Phe 66, Tyr 124, Tyr 126, His 104.	Reduction of the RNA-binding capacity of NP	Cell based infection	10 μ M	[36]
(-)-catechin gallate	SARS-CoV	N/A	Attenuation of NP binding affinity to RNA oligonucleotide	<i>In vitro</i> assay, a nanoparticle-based RNA oligonucleotide biochip system.	*IC ₅₀ of 0.05 μ g/mL	[45]
(-)-galocatechin gallate.	SARS-CoV	N/A	Attenuation of NP binding affinity to RNA oligonucleotide	<i>In vitro</i> assay, a nanoparticle-based RNA oligonucleotide biochip system.	*IC ₅₀ of 0.05 μ g/mL	[45]
P3, 5-benzyloxygr- amine	MERS-CoV	Monomer1: W43, N66, N68, S69, T70, N73, F135; Monomer2: V41, G104, T105, G106, A109, T137	Resulting in abnormal N protein oligomerization	Cell based infection	#EC ₅₀ of 32.1 μ M	[48]
ZINC00003118440, theophylline derivatives	SARS-CoV-2	Most potent hits: Gln72, Val73, Pro74, Asn76, Thr136, Thr166	Possible inhibitors of RNA-NP NTD interaction	Molecular dynamics simulation	N/A	[50]
ZINC0000146942, pyrimidone derivatives	SARS-CoV-2	Most potent hits: Gly70, Val73, Gln84	Possible inhibitors of RNA-NP NTD interaction	Molecular dynamics simulation	N/A	[50]
5817 (from Asinex databases)	SARS-CoV-2	Most potent hits: Ala55, Arg149, Asn77, Asn153, Asn154	Interaction with NP NTD	Molecular dynamics simulation	N/A	[51]
6799 (from Asinex databases)	SARS-CoV-2	Most potent hits: Ala55, Arg107, Asn75, Asn153, Asn154	Interaction with NP NTD	Molecular dynamics simulation	N/A	[51]
Zidovudine (from PubChem database)	SARS-CoV-2	Most potent hits: Ala55, Asn75, Asn77, Arg 107, Thr148, Asn150, Asn153, Asn154,	Interaction with NP NTD	Molecular dynamics simulation	N/A	[51]

*IC₅₀, half maximal inhibitory concentration.# EC₅₀, half maximal effective concentration.

demonstrated that 1,286 compounds in the FDA-approved drug database interacted with the RNA-binding region of the NP [54]. Three of the top-scoring compounds, trichlormethiazide, D-(+) biotin, and glutathione reduced free acid, presented an inhibitory effect on the binding between NPs and nucleic acids *in vitro*, indicating that these compounds interfere with the RNA-binding activity of NPs. The inhibitory activity of both trichlormethiazide and D-(+) biotin against PEDV occurred at 0.094 mg/mL with significant cell death observed above this concentration. Although glutathione reduced free acid had a minor cytotoxic effect on Vero cells, inhibition of PEDV replication required a higher concentration (1.5 mg/ml). According to the NP-compound docking result, a potential mechanism shared among these three compounds is via interacting with His74 of NPs by H-bonds or ammonium ions [54].

In addition to chemical compounds, another study revealed that RNA interference targeting PEDV NPs could be effective in inhibiting virus replication. Shi *et al.*, analyzed 25 sequences of PEDV NPs from different countries and designed short hairpin RNAs (shRNAs) against regions conserved between classical and emerging PEDV [55]. Cell viability was not affected by transfection of plasmids expressing the shRNAs. Two shRNAs targeting the NTD and one shRNA targeting the CTD of NPs showed dose-dependent inhibitory effects on both NP expression and PEDV replication in swine intestine epithelial cells. Particularly, all of the designed shRNAs had comparable antiviral function against the classical CV777 strain and the emerging LNCT2 strain [55].

During virus infection, innate immune pathways are quickly stimulated, and specific antiviral proteins can be upregulated to counter pathogens at the first line. Bone marrow stromal cell antigen 2 (BST2; tetherin; CD317; HM1.24), an IFN-inducible antiviral protein, has been shown to inhibit the release of budding virions from cells infected with either DNA or RNA viruses, including PEDV

[56]. Upregulation of BST2 was identified in Vero and porcine kidney cells, which is correlated to the inhibition of PEDV replication. Multiple mechanisms through which BST2 targets the NP of PEDV have been reported. BST2 physically interacted with the full-length NP at its glycosyl-phosphatidylinositol domain; therefore, the integrity of NP is important to the stability of the BST2-NP complex. Furthermore, the overexpression of BST2 significantly reduced the production of PEDV NP by enhancing protein degradation via autophagy [56].

Several monoclonal antibodies against the PEDV NP have been utilized to develop methods for early detection and characterization of NP epitopes. The specificity of these antibodies has been investigated and, based on the results of NP cross-reactivity between PEDV and TGEV, precise diagnostic assays for differentiating various swine coronavirus infections with similar clinical symptoms have been established [57,58]. However, these monoclonal antibodies have not been tested for their therapeutic or prophylactic potential in a coronavirus infection. Small molecule inhibitors, such as benzothiazolium and cardenolides, had anti-TGEV activity in swine testicle cells [59,60]. TGEV NPs were used as a read-out in the *in vitro* assay, and dose-dependent effects of the compounds were related to NP expression. Both NP expression and virus replication were negatively correlated with the concentration of the compounds in cells [59]. Nonetheless, more detailed experiments are required to confirm whether these compounds directly influence the function of TGEV NPs.

5.2. Avian coronaviruses

Gammacoronaviruses are mainly associated with avian and marine hosts. However, the avian CoV, IBV, is one of the major causes of highly contagious respiratory diseases in domestic fowl. Turkey

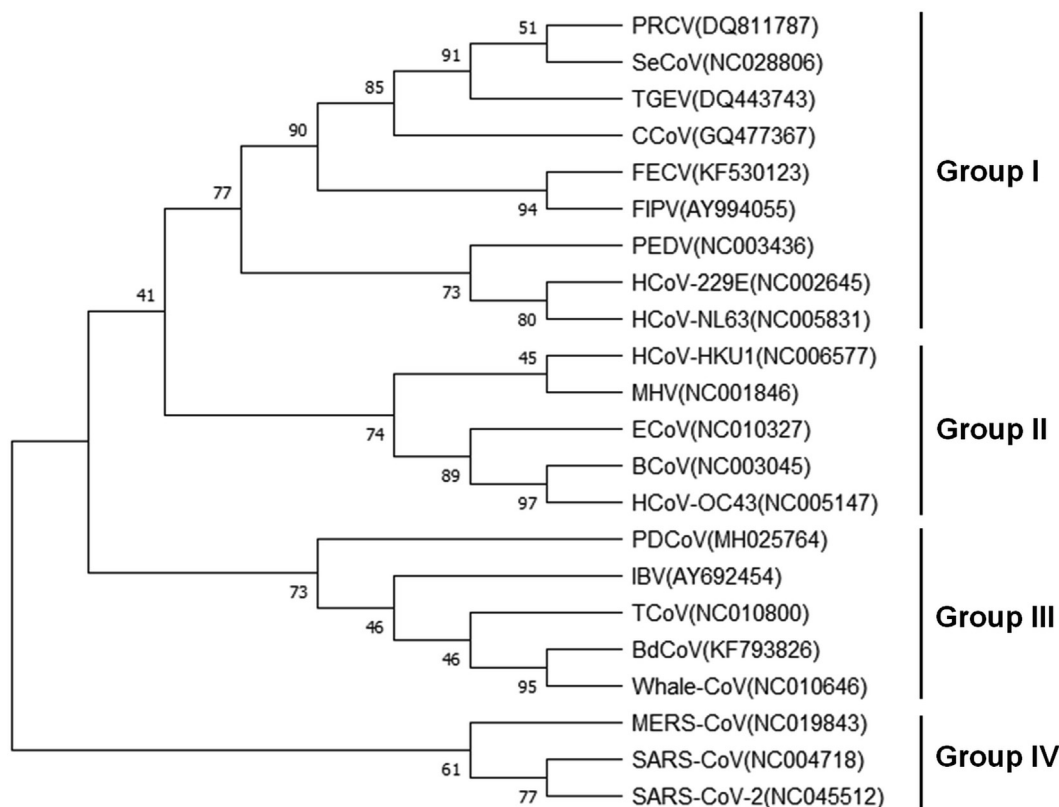


Fig. 2. Phylogenetic analysis of NP of coronaviruses. Full length NP sequences were retrieved from the GenBank database and the Maximum Likelihood method was performed to infer the evolutionary history. The bootstrap consensus tree from 500 replicates was shown and the branches were labelled with the percentage of replicate trees in the bootstrap test. PRCV, porcine respiratory coronavirus; SeCoV, swine enteric coronavirus; TGEV, transmissible gastroenteritis virus; CCoV, canine coronavirus; FECV, feline enteric coronavirus; FIPV, feline infectious peritonitis virus; PEDV, porcine epidemic diarrhea virus; HCoV-229E, human coronavirus 229E; HCoV-NL63, human coronavirus NL63; HCoV-HKU1, human coronavirus HKU1; MHV, mouse hepatitis virus; ECoV, Equine coronavirus; BCoV, bovine coronavirus; HCoV-OC43, human coronavirus OC43; PDCoV, porcine deltacoronavirus; IBV, infectious bronchitis virus; TCoV, turkey coronavirus; BdCoV, bottlenose dolphin coronavirus; Whale-CoV, whale coronavirus; MERS-CoV, Middle East respiratory syndrome coronavirus; SARS-CoV, severe acute respiratory syndrome coronavirus; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

coronavirus (TCoV) also belongs to this genus, but there is relatively limited antiviral information regarding it. Two different groups of small molecules have been found to interact with the RNA-binding domain of IBV NP protein. Eucalyptol, also named 1, 8-cineole, is an achiral aromatic component found in many plants, such as Eucalyptus and Salvia leaves. Its anti-inflammatory and apoptosis-inducing effects have been well-documented. The maximum noncytotoxic concentration of eucalyptol on Vero cells has been determined to be 3.90 mM, with an IC_{50} of 0.61 mM against IBV infection [61]. Eucalyptol has different inhibitory effects at various stages of IBV infection. The highest percent of inhibition, 82.61%, occurred when cells were treated with eucalyptol during the replication period of IBV infection. When the virus was pre-treated with eucalyptol prior to infection, replication was inhibited by 61.68%. However, treating cells with eucalyptol prior to infection or during the adsorption period only provided about 10% and 5% inhibition of virus replication, respectively. An *in silico* NP and eucalyptol model demonstrated that the H-bond formed between the oxygen atom in the core of eucalyptol and Tyr92 at the N-terminus of the IBV NP. Protein spatial analysis revealed that eucalyptol strongly associated with 5 active residues in the RNA-binding domain of IBV NPs (Tyr92, Pro134, Phe137, Asp138 and Tyr140). These residues are fully or partially conserved in various IBV strains, indicating that eucalyptol may have cross-strain anti-IBV activity. (–)-Pinenes also directly target the RNA-binding domain of IBV NPs, and are found in two isoforms as the major component of pine tree oil, or turpentine [62]. Its antimicrobial activities have been reported. Both the cyclohexene ring of (–)-

α -pinene and (–)- β -pinene bind strongly to residues Tyr92 and Pro134 of IBV NPs. (–)- α -pinene had slightly higher bioactivity with the IBV NP compared to (–)- β -pinene, as the interaction energy of the former was $-36.83 \text{ kcal mol}^{-1}$ and $-35.59 \text{ kcal mol}^{-1}$ for the latter. Consistently, IC_{50} of (–)- α -pinene against IBV replication was lower (0.98 mM) than that of (–)- β -pinene (1.32 mM).

In addition to interfering with the function of the RNA-binding domain of IBV NPs, Scott *et al.*, found that a hammerhead ribozyme can specifically cleave NP mRNA of IBV [63]. Hammerhead ribozymes, the catalytic RNA, constitute of a catalytic domain that cleaves target RNA and the flanking sequences are responsible for the specificity of the ribozyme. As revealed by both gel electrophoresis and quantitative polymerase chain reaction, the RNA of IBV NPs was specifically cleaved by a hammerhead ribozyme that leads to decline of NP RNA level. In addition to IBV, hammerhead ribozyme strategy has successfully restricted the mouse coronavirus MHV and other viruses in different viral families by targeting various genes, including *Arenaviridae* (to cleave the S genomic RNA of lymphocytic choriomeningitis virus) [64], *Birnaviridae* (RNA polymerase gene of infectious bursal disease virus) [65], *Paramyxoviridae* (nucleocapsid mRNA of mumps virus) [66], *Picornaviridae* (5' untranslated region of hepatitis C virus) [67] and *Retroviridae* (5' untranslated region of HIV-1) [68].

5.3. Feline coronaviruses

Feline coronaviruses are classified as *Alphacoronavirus*; other members of this subgroup include CCoV and TGEV. Currently,

Table 2
Therapeutics against NPs of domestic animal CoVs.

Compounds	Virus	Binding sites	Mechanism	Assay system	Dose	Reference
Trichlormethiazide	PEDV	His74, Tyr76, Arg113	Interference with RNA-binding activity of NP	Cell based infection	0.094 mg/ mL	[54]
D-(+) biotin	PEDV	Ser 17, His74	Interference with RNA-binding activity of NP	Cell based infection	0.094 mg/ mL	[54]
Glutathione reduced free acid	PEDV	Tyr19, Ser17, His74, Arg113	Interference with RNA-binding activity of NP	Cell based infection	1.5 mg/ mL	[54]
shRNA-N307	PEDV	NP gene sequence: 307–327	Inhibitory effect on NP production	Cell based infection	1–4 µg/ 5x10 ⁴ cells	[55]
shRNA-N463	PEDV	NP gene sequence: 463–483	Inhibitory effect on NP production	Cell based infection	1–4 µg/ 5x10 ⁴ cells	[55]
shRNA-N1071	PEDV	NP gene: 1071–1091	Inhibitory effect on NP production	Cell based infection	1–4 µg/ 5x10 ⁴ cells	[55]
BST2	PEDV	Full length NP	Interaction with NP NTD	Cell based infection	N/A	[56]
Eucalyptol	IBV	Tyr92, Pro134, Phe137, Asp138, Tyr140	Interference with RNA-binding activity of NP	Cell based infection	3.90 mM	[61]
(-)-α-pinene	IBV	Ala33, Ser34, Gln37, Tyr92, Pro134, Phe137, Asp138, Gln139, Gly147, Pro149	Interaction with NP RBD	Cell based infection	7.88 mM	[62]
(-)-β-pinene	IBV	Ala33, Ser34, Gln37, Tyr92, Pro134, Phe137, Asp138, Gln139, Gly147, Pro149	Interaction with NP RBD	Cell based infection	6.09 mM	[62]
Hammerhead ribozyme	IBV	NP mRNA	Cleavage of NP mRNA	<i>In vitro</i> assays	0.5–10 µM	[63]
siRNA NP (N1)	FIPV	FIPV genome (27112–27130)	Reduction of viral genomic RNA	Cell based infection	5 nM	[70]
siRNA NP (N2)	FIPV	FIPV genome (27885–27837)	Reduction of viral genomic RNA	Cell based infection	100 nM	[70]
siRNA-N	FIPV	FIPV genome (27507–27531)	Reduction of viral genomic RNA	Cell based infection	100 nM	[71]

two feline coronaviruses are circulating worldwide: feline enteric coronavirus (FECV) and FIPV [69]. FECV is prevalent in domestic cats and usually causes mild or subclinical symptoms. In contrast, FIPV, derived from parental FECV with specific mutations, is highly virulent and causes fatal peritonitis in cats of any age, but especially between the ages of 4 to 16 months [69]. To date, there is no effective treatment for FIPV, and mortality is extremely high. Several groups have identified small molecules with anti-FIPV potential, such as small interfering RNA (siRNA) which inhibits FIPV infection *in vitro* using the FIPV nucleocapsid gene or protein as a read-out; nevertheless, whether these drugs directly target with NPs has been approved yet. According to two different studies, siRNA targeting the FIPV NP gene is an efficient strategy to reduce the copy number of genomic and messenger FIPV RNA, viral protein expression, and production of viral progeny [70,71]. The potency of siRNA specific to the NP gene is comparable to that of siRNA targeting a 5' leader sequence essential to controlling coronavirus gene expression. Importantly, siRNA is effective against FIPV even at the lowest tested concentration (5 nM), and can be used to inhibit FIPV replication within a broad range of multiplicity of infection [70]. Combinations of siRNAs specific to different regions of the FIPV genome resulted in more effective viral inhibition [71]. The selection of these siRNA was based on homology between various FIPV strains, suggesting the possibility of a cross-strain inhibitory effect.

At present, RNA interference strategy has been shown to inhibit PEDV, IBV and FIPV infection. However, it is the only approach to restrict FIPV replication in cells. It is worthy of noting that all of validations were performed in cells or by *in vitro* system. Further investigation of safety and effect of indicated compounds are required in animal models. A brief summary of inhibitors against domestic animal CoVs was listed in Table 2.

6. Concluding remarks

As NPs share several conserved residues and structures across a large variety of coronavirus strains, they are a promising target for

drug discovery with the potential to develop cross-strain activity. Most studies focused on finding compounds that interact with RNA-binding domain at the N-terminus, which specifically suppresses the efficiency of coronavirus genome replication and virion assembly. Virtual screening of high resolution structural information of native interacting interfaces against multiple drug libraries is the main method of discovering novel therapeutic agents. Alternatively, potential compound candidates can be selected according to structure-based stabilization of non-native protein–protein interactions. As such, specific ligands may enhance or inhibit the interaction between NP molecules, leading to abnormal oligomerization and therefore, anti-coronavirus activity. Although these data are all preclinical and more detailed experiments and analysis of NP-compound structures are required, this review could be a basis for developing effective treatments for coronavirus infection.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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