



Nucleoside analogues for the treatment of coronavirus infections

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Recent outbreaks of SARS-Coronavirus and MERS-Coronavirus (CoV) have heightened awareness about the lack of vaccines or antiviral compounds approved for prevention or treatment of human or potential zoonotic CoVs. Anti-CoV drug development has long been challenged by the activity of a 3' to 5' proofreading exoribonuclease unique to CoVs. Recently, a promising nucleoside analogue with broad-spectrum activity against CoVs has been identified. This review will discuss progress made in the development of antiviral nucleoside and nucleotide analogues targeting viral RNA synthesis as effective therapeutics against CoV infections and propose promising strategies for combination therapy.

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Introduction

Coronaviruses (CoVs) are a family of enveloped viruses containing a positive-sense RNA genome. CoVs belong to the family *Coronaviridae* within the order *Nidovirales*. The *Coronaviridae*, henceforth referred to as CoVs, infect a broad range of vertebrates including mammals and birds. Of the six CoV strains isolated from humans, four strains (HCoV-229E, HCoV-NL63, HCoV-HKU1, and HCoV-OC43) cause a usually mild self-limiting upper respiratory infection accounting for an estimated 15–29% of common colds [1]. Two other human CoV strains have produced more serious respiratory disease epidemics in recent years. Severe acute respiratory syndrome (SARS)-CoV originated in China in 2002 and 2003 and spread to a total of 37 countries, causing more than 8000 cases of

SARS with an estimated fatality rate of 9% [2]. The cause of an ongoing epidemic of Middle East respiratory syndrome, MERS-CoV was first identified in Saudi Arabia in 2012 and to date has caused more than 2374 cases in 27 countries with a fatality rate >34% [3,4]. The use of human host cell receptors by both human and bat CoVs supports predictions of significant risk for emergence of new potential pandemic zoonotic CoVs [5,6]. Notably, no vaccines or antiviral compounds are approved for prevention or treatment of human or potential zoonotic CoVs. Emerging CoVs have been accorded priority status by WHO and government agencies for development of prevention and treatment strategies due to severity of these infections and credible pandemic potential [7–9]. This review will focus on opportunities for and challenges to development of antiviral nucleoside and nucleotide analogues targeting viral RNA synthesis as effective therapeutics against CoV infections.

CoV antiviral strategies

Drugs currently under investigation for use against CoV disease include monoclonal antibodies; direct-acting antivirals (DAAs) including as protease, helicase, and polymerase inhibitors; and immunomodulators such as interferons and corticosteroids. Challenges to CoV antiviral development are both general to RNA viruses and specific to CoVs. The replication of positive-sense RNA virus genomes is generally characterized by high error rates, high viral yields, short replication times, and abundant homologous and nonhomologous recombination [10]. In consequence, ‘viral swarms’ are generated which consist of a diverse population of genome mutants with varying degrees of fitness. This genetic plasticity challenges development of broadly useful antivirals by enabling rapid development of drug resistance while preserving overall viral fitness.

Development of nucleoside analogue inhibitors of CoVs is further hampered by the novel RNA-dependent RNA proofreading activity of CoV nonstructural protein 14, a 3' to 5' exoribonuclease (nsp14-ExoN), which confers up to 20-fold increase in replication fidelity compared with other RNA viruses. The nsp14-ExoN activity is responsible for native CoV high resistance to many nucleoside analogues including ribavirin and 5-fluorouracil [11–13]. Nevertheless, the recent development of nucleotide and nucleoside analogue inhibitors with a high barrier for resistance, broad-spectrum activity against multiple CoVs, and the ability to inhibit WT CoVs in the presence

of nsp14-ExoN holds promise for the treatment of CoV disease.

Nucleotide and nucleoside analogue inhibitors for the treatment of CoV infections

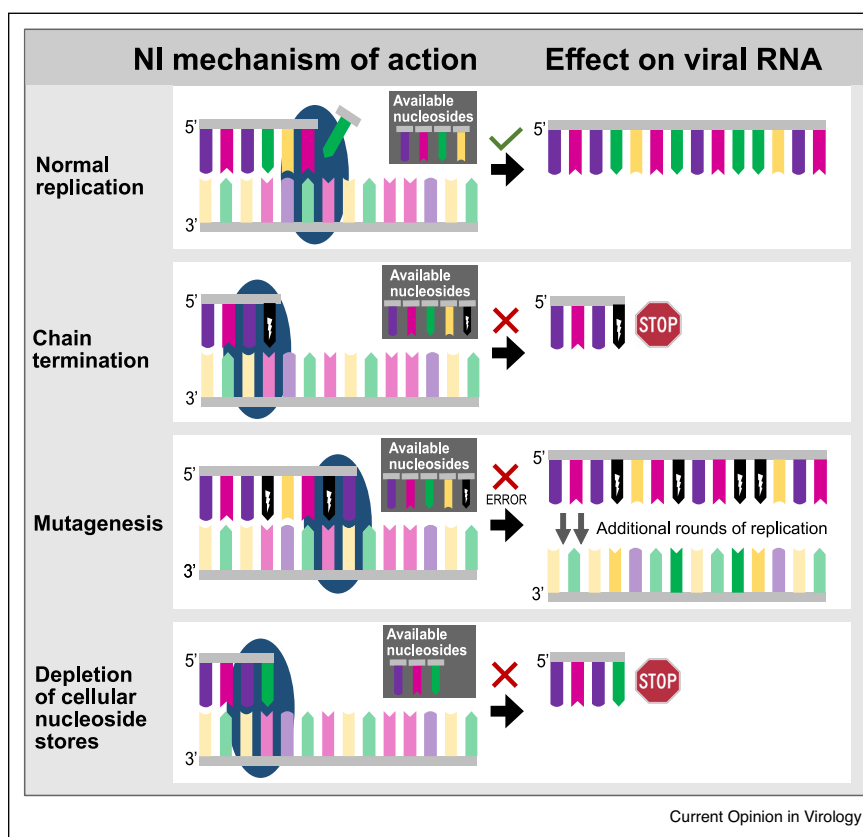
Nucleotide and nucleoside analogue inhibitors, hereafter abbreviated NI, are chemically synthesized analogues of purines and pyrimidines in which the heterocyclic ring or sugar moiety has been altered. Currently used to treat both chronic and acute viral infections, NIs are administered as nucleotide or nucleoside precursors or prodrugs, which are metabolized by host or viral kinases to their active triphosphate once inside the cell.

NIs exert inhibitory effects on viral replication by one or more non-mutually exclusive mechanisms (Figure 1). First, mis-incorporation of foreign nucleotides in replicating viral genomes may cause chain termination and disrupt subsequent replication or transcription. Chain termination may be immediate (obligate) or may occur following a limited extent of continued RNA or DNA synthesis (non-obligate). Second, NIs may incorporate into elongating nucleotide chains,

mispairing with and/or substituting natural nucleotides, thereby introducing mutations that potentially impair RNA synthesis, structure, or RNA–protein interactions or protein functions. Accumulation of mutations and loss of virus viability are referred to as lethal mutagenesis and error catastrophe [14]. Through these mechanisms, NIs alter the genetic makeup of the virus, leading to a decrease in viral fitness with every consecutive replication cycle. Finally, NIs may cause depletion of pools of naturally occurring nucleotides by mimicking [15].

NIs demonstrate a relatively high barrier to resistance emergence because the structural conservation of the binding site of their polymerase targets is high among virus families, and resistance mutations generally incur a fitness cost for the enzyme and the virus [16]. For CoVs, amino acid conservation of the viral RdRp ranges from 70 to near 100% and is maintained across genera, suggesting NIs could potentially serve as broad-spectrum inhibitors of CoV infection [17^{**}]. However, proofreading activity of nsp14-ExoN activity protects CoVs from many NIs effective against other RNA viruses [12,18]. To effectively inhibit CoVs, an NI needs to either evade recognition by

Figure 1



Mechanisms of inhibition by nucleoside and nucleotide analogues. Schematic representation illustrates normal replication by the RdRp (blue sphere), premature chain termination caused by an obligate chain terminator, reduced replication fidelity due to mutagen incorporation, and depletion of pools of naturally occurring nucleotides.

ExoN or undergo uptake into the elongating strand at a rate exceeding ExoN excision kinetics. We will next discuss several antiviral NIs described in the literature and evidence supporting their efficacy against CoVs.

Ribavirin (1- β -D-ribofuranosyl-1, 2,4-triazole-3-carboxamide) is a guanosine analogue with broad-spectrum antiviral activity against RNA viruses. It is used to treat hepatitis C and E virus, respiratory syncytial virus, Lassa virus, and hantavirus infections. In its monophosphate form, ribavirin interactions with inosine monophosphate dehydrogenase (IMPDH), a host enzyme vital to nucleotide biosynthesis, result in decreased guanosine production leading to inhibition of viral RNA synthesis through depletion of cellular GTP pools [19]. Furthermore, incorporation of its triphosphate form by the viral polymerase leads to lethal mutagenesis [20]. The proofreading activity of ExoN leaves CoVs impervious to doses of ribavirin that inhibit other viruses effectively [12]. Although high dose ribavirin showed partial inhibition of SARS-CoV and MERS-CoV replication *in vitro* (Table 1) [18,21], drug treatment increased viral load and exacerbated disease in a mouse model of SARS-CoV disease [22]. Ribavirin treatment did not improve the clinical outcome of SARS-CoV disease in human subjects and caused significant toxicity [23,24]. Synergistic activity against MERS-CoV of ribavirin combined with IFN α 2b was observed *in vitro* and in rhesus macaques, suggesting that IFN increases the potency of ribavirin at lower, more tolerable concentrations [25,26]. However, five critically ill MERS-CoV-positive patients who were treated with a combination of ribavirin and IFN α 2b showed no clinical improvement [27]. Treatment of 20 MERS patients with a combination of ribavirin and IFN α 2a showed significantly

improved survival at 14 days but not at 28 days [28], whereas treatment of MERS patients with a combination of IFN α 2a or IFN β 1a and ribavirin yielded no survival benefit in another study [29]. Thus, although ribavirin shows some efficacy *in vitro*, it does not provide clinical benefit to humans with SARS-CoV or MERS-CoV infections.

Remdesivir (Gilead Science (GS)-5734) is a phosphoramidate prodrug of the adenosine NI GS-441524 which is effective against filoviruses, pneumoviruses, and paramyxoviruses and is currently in a Phase I dose-escalation trial for Ebola virus infection [30]. Biochemical studies indicate that remdesivir acts as a non-obligate chain terminator [31]. Remdesivir is effective against a broad spectrum of human and pre-epidemic zoonotic CoVs and potentially inhibits replication of SARS-CoV and MERS-CoV in primary human airway epithelial cultures (Table 1) [17^{**},32^{**}]. Mice infected with a lethal dose of SARS-CoV displayed less weight loss, lower lung viral titer, and reduced lung pathology following prophylactic or therapeutic administration of remdesivir [17^{**}]. A partial resistance phenotype was attributed to two mutations in the viral RdRp, suggesting remdesivir acts on this target [32^{**}]. Importantly, the fitness and virulence of remdesivir-resistant SARS-CoV was reduced compared to WT SARS-CoV, indicating the barrier to remdesivir resistance is high [17^{**}]. Increased potency of remdesivir against CoV lacking ExoN catalytic activity suggests that the drug is sensitive to proofreading by ExoN, albeit to a markedly lower extent than other NIs [32^{**}]. This raises important questions about the mechanism of CoV inhibition by remdesivir, which may be recognized by ExoN but removed less efficiently or may be less visible to ExoN compared with other NIs. Studies probing the interactions between remdesivir and the CoV replication machinery will likely yield crucial insights into how this NI circumvents or overcomes CoV proofreading activity, which can in turn be applied to modeling the development of new NIs and enhancing potency of existing NIs.

Table 1

Nucleoside analogues with demonstrated activity against human CoVs

Nucleoside analogue	EC50 or IC50	Citation
Ribavirin	50–819 μ M (SARS-CoV) 20 μ M (MERS-CoV)	[21,51]
Remdesivir	0.07 μ M (SARS-CoV) 0.03–0.07 μ M (MERS-CoV) 0.03 μ M (MHV)	[17 ^{**} ,32 ^{**}]
β -D-N4-hydroxycytidine	0.4 μ M (HCoV-NL63) 5 μ M (SARS-CoV)	[36,38]
BCX4430	68.4 μ M (MERS-CoV) 57.7 μ M (SARS-CoV)	[31]
Gemcitabine hydrochloride	1.22 μ M (MERS-CoV) 4.96 μ M (SARS-CoV)	[40]
6-Azauridine	0.03 μ M (HCoV-NL63)	[36]
Mizoribine	13.5 μ M (MERS-CoV) 62 μ M (SARS-CoV)	[18]
Acyclovir fleximer	23 μ M (MERS-CoV) 8.8 μ M (HCoV-NL63)	[41]

Beta-D-N4-hydroxycytidine (NHC): NHC is a cytidine analogue that has demonstrated potent, broad-spectrum antiviral activity against Venezuelan equine encephalitis virus (VEEV), respiratory syncytial virus (RSV), influenza A virus (IAV), influenza B virus (IBV), chikungunya virus (CHIKV), and CoVs (Table 1). NHC exerts its antiviral effect primarily through mutagenesis of viral RNA [22,33,34,35^{*},36]. Serial passaging in the presence of NHC led to low level resistance for VEEV but not RSV, IAV, and bovine viral diarrhea virus, thus indicating a high resistance barrier [33,35^{*},37]. Potent anti-CoV activity of NHC was demonstrated for SARS-CoV and HCoV-NL63 [36,38]. Although the mechanism of CoV inhibition has not been determined, micromolar-range EC₅₀s suggests that like remdesivir, NHC may also have a novel way of interacting with the CoV replicase. The

potent, broad-spectrum antiviral activity exhibited by NHC warrants further investigation into use of NHC for the treatment of CoV infections, either alone or in combination with other DAAs and immunomodulators.

Other NIs with *in vitro* activity against CoVs and low cytotoxicity include the adenosine analogue BCX4430, which has broad spectrum activity against positive and negative sense RNA viruses and has shown antiviral activity against MERS-CoV and SARS-CoV [31,39]; the deoxycytidine analogue gemcitabine hydrochloride, a chemotherapy drug that inhibits SARS-CoV and MERS-CoV [40]; the uridine analogue 6-azauridine with activity against HCoV-NL63 [36]; and the immunosuppressant imidazole nucleoside mizoribine which inhibits SARS-CoV (Table 1) [18]. Flex-base modification of the guanosine analog acyclovir (acyclovir fleximer) yielded activity against HCoV-NL63 and MERS-CoV (Table 1) [41]. More research into the efficacy, potency, and mechanism of CoV inhibition is necessary to determine whether further development of these compounds as CoV antivirals is warranted.

Conclusions and outlook

As with SARS-CoV and MERS-CoV, new zoonotic CoVs likely will emerge from divergent virus pools in animal reservoirs. It is therefore critical to develop broad-spectrum anti-CoV strategies aimed at multiple conserved targets and functions. Despite high conservation of the viral RdRp and low tolerance for mutations at key residues, resistance against NIs due to mutations in the viral RdRp has been observed in CoVs and other RNA viruses [32[•],42,43[•],44,45]. Treatment with combinations of potent anti-CoV NIs could increase the barrier to resistance and enhance efficacy, especially if additive or synergistic interactions occur. Moreover, a therapeutic regimen that combines drugs with distinct modes of action or which interfere at different steps in the viral replication cycle could simultaneously increase antiviral potency, broaden the activity spectrum, and reduce the emergence of drug resistance against CoVs. Classes of candidate companion compounds include NIs, helicase inhibitors, protease inhibitors, monoclonal antibodies, viral entry inhibitors, and DEDDh family exoribonuclease inhibitors with potential activity against ExoN [46,47[•],48], the latter deserving special interest. DEDDh inhibitors aurantricarboxylic acid and pontacyl violet 6R effectively inhibited Lassa virus NP exonuclease in a proof-of-concept biochemical assay [48]. The structural and functional conservation across CoV family members and the absence of redundant functions elsewhere in the genome makes ExoN a potential Achilles' heel. Combining compounds that inhibit ExoN activity with one or more NIs would simultaneously reduce CoV replication fidelity, boost the potency of the NI, mitigate selective pressures leading to drug resistance, and ultimately attenuate viral disease.

Finally, both SARS-CoV and MERS-CoV infections spur exuberant host inflammatory responses that rapidly progress toward severe immunopathology, the most probable driver of morbidity and death rather than direct viral damage to pulmonary tissues [49]. This potentially limits the therapeutic window for DAAs. Thus, combinations of DAAs and targeted immunomodulators may be necessary to halt lethal progression of immunopathology and extend the therapeutic window for intervention. A multicenter, placebo-controlled, double-blind randomized trial (MIRACLE: NCT02845843) is currently in progress to determine the efficacy of combining the immunomodulator IFN β 1b with lopinavir-ritonavir, a protease inhibitor cocktail used to treat HIV that also inhibits MERS-CoV *in vitro* [50]. Adequately controlled prospective studies like MIRACLE are urgently needed to assess efficacy of candidate CoV drugs. These studies should include compounds that have demonstrated broad-spectrum *in vivo* efficacy against CoVs, such as remdesivir, knowing that additional novel zoonotic CoVs are an inevitable future occurrence.

Conflict of interest statement

Nothing declared.

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