Lethal mutagenesis as an antiviral strategy

Lethal mutagenesis of RNA viruses is a viable antiviral strategy but has unknown risks

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Viruses depend on the host cell to carry out much of their replication, with each offering only a few virus-specific targets for the development of antiviral therapies. This makes the development of broadly active antivirals difficult to conceptualize. Numerous RNA viruses—including severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), Zika virus, and Chikungunya virus—have led to recent epidemics, highlighting the need for effective antiviral drugs that can be enlisted quickly. Some years ago, a broadly applicable antiviral strategy was proposed in which a slight increase in the error rate of a rapidly replicating RNA virus would overwhelm the capacity to remove deleterious mutations, driving the viral population to extinction; this strategy is called lethal mutagenesis (*1*). Although the antivirals ribavirin and favipiravir were developed with this strategy in mind, the recent development of the much more potent molnupiravir to treat SARS-CoV-2 highlights the unknown risks to the host that this strategy entails.

The genome size of an organism is inversely related to the error rate during replication, and this holds true for small RNA viruses with genomes of 7 to 30 kb (*2*). For RNA viruses, this translates into one nucleotide substitution for every two to three genomes synthesized. Most mutations are deleterious, but a subset of mutations will give rise to potentially useful phenotypic diversity, which may undergo selection. Lethal mutagenesis is a universal antiviral strategy for RNA viruses (especially those that cause acute disease) because they all have the same vulnerabilities of small genomes and rapid replication, making them highly sensitive to an increased mutation rate.

The strategy for increasing the rate of new mutations in RNA viruses is to design ribonucleoside analogs that can be metabolized to ribonucleoside triphosphates in cells and then be incorporated into the viral genome during viral RNA synthesis. The design of the analog allows the base portion of the ribonucleotide to base pair ambiguously during subsequent RNA synthesis. Thus, once incorporated into viral RNA, the analog will base pair with one of several natural nucleotides during RNA synthesis, leading to a mutation. RNA viruses synthesize complementary plus and minus strands of RNA during viral replication and do this multiple times. For example, it is estimated that the poliovirus RNA genome undergoes five consecutive rounds of replication within a cell before new virus particles are released (*3*). As the viral RNA genome is amplified in the cell, the effects of the mutagen are concentrated in the viral genome.

The first ribonucleoside analog that was identified as capable of inducing mutations in an RNA virus was the purine analog ribavirin, which forms base pairs as either adenosine or guanosine when used at high concentrations in human cells in vitro (*4*). Ribavirin has pleotropic effects on the cell, and its limited antiviral effect in vivo is by an uncertain mechanism (*5*). Favipiravir is a base analog that is metabolized to a ribavirin-like molecule in the cell. It is approved for use against influenza virus infection in Japan, and it has been shown to be antiviral and mutagenic against SARS-CoV-2 when used at high doses in an animal model (*6*, *7*). Favipiravir is now being evaluated in multiple human trials to treat COVID-19.

A significantly more potent antiviral drug that mediates lethal mutagenesis has recently come to the forefront as a potential antiviral in the current SARS-CoV-2 pandemic—molnupiravir (*8*, *9*). This is an orally available 5′-isobutyl form of the cytidine analog β-D-N^4-hydroxycytidine (NHC) (*10*). This molecule contains an additional oxygen atom in the extra-ring amino group at position four of the cytidine base. In this position, the oxygen destabilizes a hydrogen atom, also bound to this extra-ring nitrogen, leading to migration back and forth with the ring position three nitrogen; this changes the base-pairing properties back and forth between uridine and cytidine (*11*, *12*) (see the figure). In uridine, position four in the ring of the base has an extra-ring oxygen as a carbonyl, suggesting that RNA synthesis is relatively insensitive to the chemical composition at this position (aside from its role in base pairing). This highlights why NHC should be readily metabolized by the cell. In a cell culture–based assay, NHC was 100 times more potent as an inhibitor of SARS-CoV-2 than ribavirin or favipiravir (*13*). Molnupiravir was efficacious in mouse models of respiratory SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV) infection (*9*), consistent with NHC having broad antiviral activity (*10*).

A recently reported clinical trial of molnupiravir showed a 30% reduction in hospitalization when people with symptomatic SARS-CoV-2 infection (and at risk for more serious disease) were treated with molnupiravir within the first 5 days of symptoms (*14*). Based on these results, the US Food and Drug Administration (FDA) has approved an emergency use authorization (EUA) for molnupiravir to treat symptomatic SARS-CoV-2 infections. Molnupiravir has also been approved for the treatment of COVID-19 in the United Kingdom, and there are expectations that it will be made widely available around the world.

However, the antiviral strategy of lethal mutagenesis comes with a cautionary note. Ribonucleosides must be phosphorylated to the 5′-triphosphate form to be substrates for RNA synthesis (host or viral). Ribonucleosides synthesized by the host cell are formed as the 5′-monophosphate. Ribonucleoside analogs enter this biosynthetic pathway through phosphorylation by a salvage kinase to form the 5′-monophosphate (see the figure). The ribonucleoside 5′-monophosphate is phosphorylated to the ribonucleoside 5′-diphosphate and then to the 5′-triphosphate (now ready for RNA synthesis). The ribonucleoside 5′-diphosphate is the obligatory intermediate in this pathway, which creates a potential problem. Ribonucleoside 5′-diphosphate is also the obligatory intermediate in the synthesis of the 2′-deoxyribonucleoside 5′-diphosphate that is on the pathway to form 2′-deoxyribonucleoside 5′-triphosphates, which are used in DNA synthesis. The enzyme ribonucleotide reductase (RNR) is responsible for this reaction. Thus, there is a clear metabolic pathway for a mutagenic ribonucleoside analog to become a precursor for host DNA synthesis.

Molnupiravir was shown to be positive in the bacterial Ames test (an assay that measures mutagenic potential), where two animal model assays of mutagenic potential were largely negative, leading the FDA to state in the EUA fact sheet that "molnupiravir is low

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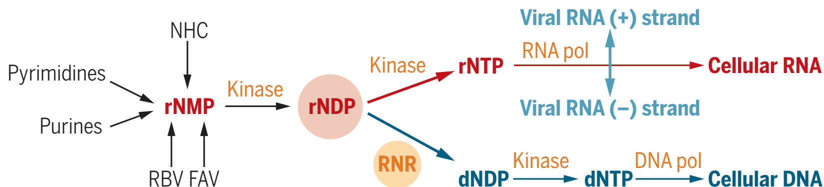
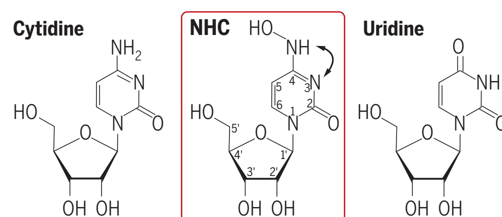
Mutagenesis with ribonucleoside analogs

Antiviral ribonucleoside analogs—such as NHC (molnupiravir), RBV, and FAV—transit the ribonucleotide biosynthetic pathway and become the substrate for host and viral RNA synthesis. They may also appear in the 2'-deoxyribonucleotide pathway owing to the activity of RNR.



Ribonucleoside analogs converge at the rNMP, which is metabolized to the rNDP and then to the rNTP to become the substrate for host and viral RNA synthesis. However, the rNDP is also the substrate for the synthesis of the DNA precursor dNDP.



Mutations are introduced during viral replication when the NHC-derived ribonucleotide in viral RNA is recognized as either cytidine or uridine owing to ambiguous base pairing.

dNDP, 2'-deoxyribonucleoside 5'-diphosphate; dNTP, 2'-deoxyribonucleoside 5'-triphosphate; FAV, favipiravir; NHC, β-D-N^4-hydroxycytidine; pol, polymerase; RBV, ribavirin; rNDP, ribonucleoside 5'-diphosphate; rNMP, ribonucleoside 5'-monophosphate; RNR, ribonucleotide reductase; rNTP, ribonucleoside 5'-triphosphate.

risk for genotoxicity" (*15*). However, the ability of the molnupiravir metabolite NHC to transit the RNR pathway was demonstrated in a cell culture–based assay of mammalian cell mutagenesis (*13*), raising questions about which assays should be used for evaluating the risk of mutagenesis in humans.

There is a gap in our knowledge in scaling short-term lab-based assays (using bacteria, animal cells, and animal models) for mutagenic activity with long-term risk to human health. Mutagens that are incorporated during cellular DNA synthesis are problematic for a developing fetus (where cells are undergoing rapid division), male germline cells (which continue to divide throughout life), and cancer risk (where the small fraction of human cells that are dividing have the potential to incorporate a mutation that could contribute to cancer development). Humans are exposed to mutagens throughout life—for example, DNA mutations are induced by x-ray imaging or during air travel—so there are levels of DNA damage that are considered to be largely inconsequential. If the molnupiravir metabolite NHC really is a mutagen in dividing animal cells, how should negative data in an animal model be interpreted? Are such negative data sufficient to ensure long-term safety in humans, or does the lack of knowledge about the link between negative results in animal assays and long-term outcomes in human health need to be acknowledged? Molnupiravir use will come with some restrictions around short-term risks associated with reproductive health, but it may take years before potential long-term risks are understood. The best outcome, which is the assumption from the negative results in animals, is that molnupiravir treatment falls within the background level of exposure to mutagens that humans already experience and tolerate. The half-life of molnupiravir metabolites in human tissue is unknown.

By definition, lethal mutagenesis will cause increased sequence diversity within the viral population. This has raised the issue of whether the intentional introduction of sequence diversity will speed up viral evolution, with the specific concern being antibody escape mutants that would undermine vaccine efforts. Adding random mutations at a density of 1 per 1000 bases of the viral genome is sufficient to reduce infectivity of the viral population in the range of 100-fold, as shown for poliovirus and SARS-CoV-2 (*4*, *13*). Treatment with molnupiravir modestly reduces the shedding of viral RNA and significantly reduces the infectiousness of SARS-CoV-2 in patients with COVID-19 (*8*, *14*). Thus, during successful treatment and clearance of the virus, the potential for evolution would appear minimal. However, for people who fail to clear the virus and maintain a persistent infection, whether treatment with molnupiravir will affect the course of viral evolution remains unknown. Similarly, attempts to treat patients with a combination of molnupiravir and the SARS-CoV-2 protease inhibitor nirmatrelvir should carefully follow any sequence changes within the viral 3CL protease coding domain to assess the potential evolution of resistance.

There is a desperate need to make efficacious SARS-CoV-2 treatments widely available, to develop new broadly active antiviral treatments to allow rapid response to new SARS-CoV-2 variants, and, more generally, to be able to respond to new RNA virus epidemics. Molnupiravir has the potential to lower the disease burden of SARS-CoV-2 infections and help contain future emerging RNA viruses. However, how can its potential long-term effects as a mutagen be assessed? The following steps are suggested: Treatment should be restricted to those who will benefit the most, such as those who cannot tolerate other available treatments, those who have a preexisting condition that enhances the risk of COVID-19, and those who are more than 50 years of age and would be less affected by a potential long-term risk of cancer or reproductive risks. A registry of a cohort of people who received molnupiravir should be kept to longitudinally monitor the frequency of cancer and other potential outcomes so that the opportunity to understand the risk (or lack thereof) associated with the use of a mutagenic ribonucleoside as an antiviral is not missed. Strategies to limit metabolism of mutagenic analogs from the ribonucleotide pool into the 2′-deoxyribonucleotide pool should be explored to limit the potential DNA mutation load in the host. In addition, the viral population diversity should be evaluated after treatment with molnupiravir in those who fail to clear the virus to see whether the treatment accelerates viral evolution. Lethal mutagenesis has the potential to be an important antiviral strategy for RNA viruses, especially in emerging infections when there is an absence of virus-specific antivirals. The potential of this strategy should be exploited, but the possible risks should be acknowledged and addressed. ■

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