**Reply to Troth et al.**

**Authors:** Shuntai Zhou[1], Collin S. Hill[1], Blaide M. D. Woodburn[1,2], Raymond F. Schinazi[3], Ronald Swanstrom[1,4*]

**Affiliations:**

[1] Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA

[2] Department of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA

[3] Laboratory of Biochemical Pharmacology, Department of Pediatrics, Emory University School of Medicine and Children's Healthcare of Atlanta, Atlanta, GA 30326, USA

[4] Department of Biochemistry and Biophysics, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA

[*] **Corresponding author:** Ronald Swanstrom. Address: Rm 22-006 Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC, 27599, USA. Email: risunc@med.unc.edu. Telephone: 919-966-5710. FAX: 919-966-8282.

**Funding**: Antiviral Drug Discovery and Development Center (NIH award U19 AI142759), and NIH award R01 AI140970 to RS.

**Conflict of interests**: The University of North Carolina is pursuing IP protection for Primer ID and R.S. has received nominal royalties.

**Key words**: SARS-CoV-2, NHC, molnupiravir, mutagenicity

We thank Troth et al. for the opportunity to extend the discussion of our data on the mutagenicity of $N^4$-hydroxycytidine (rNHC) [1]. We view our work as providing the proof-of-concept showing that as rNHC is phosphorylated to its active ribonucleoside 5'-triphosphate, the ribonucleoside 5'-diphosphate intermediate that is the immediate precursor to the ribonucleoside 5'-triphosphate also plays the equivalent role of an intermediate precursor for the synthesis of 2'-deoxyribonucleoside 5'-diphosphate (by the activity of ribonucleotide reductase). This is the normal pathway for the synthesis of DNA precursors used from bacteria to humans; thus, it should not be a question of whether the mutagenic form of dNHC as a precursor to DNA is formed, but rather what the impact is. On this point we have unpublished cell-based data supporting conversion of rNHC to dNHC, albeit at low intracellular levels. Also, the near identity of rNHC to cytidine (the addition of a single oxygen atom) makes it likely that rNHC and cytidine undergo similar metabolism in the cell.

Although we easily demonstrated the mutagenic potential of rNHC in a cell culture model, Troth et al. note their negative data using two in vivo model systems. Negative results must be viewed in the context of assay sensitivity. NHC mutagenesis will occur in dividing cells. Do the in vivo assays focus on dividing cells, and what is the limit of detection of new mutations when dividing cells are assessed? How do we scale these negative results to a human who may live for years? Mutagenesis is not an acute toxicity but, rather, would be revealed over a long period in cancer rates and germline mutations.

Troth et al. raise several questions concerning our experimental approach. First, they question our use of a 32 day drug exposure rather than 3-6 hour exposure. Since rNHC has to be taken into the cell then metabolized to become a DNA precursor, a 3-6 hour exposure would likely result in a negative result (it would likely fail as an antiviral agent also). Short exposures are relevant to chemicals that derivatize DNA, not for metabolic precursors. Thus, it is important to think about the mechanism of mutagenesis when choosing a test for mutagenic potential, both in vitro and in vivo.

We used a short term (5 day) cell toxicity/cytostatic assay. Troth et al. suggest this should have been a 32 day assessment. While we did not do this, we also did not notice a difference in growth rate in the presence of 3 µM rNHC during the multiple rounds of cell passage.

Our results using a gene knockout model demonstrate the mutagenic potential for the host, but in our adaptation of the HPRT knockout model it is difficult to establish a mutation rate given that multiple rounds of cell replication and drug incorporation occurred. Highly accurate sequencing of DNA is likely better suited to ask this question. Ultimately the rate of mutagenesis in cell culture is less relevant than the long term consequences of exposure to a mutagen during treatment.

Troth et al. pose several questions about solubility and purity.  No solubility or pH issues have been noted by visual inspection, and the concentrations chosen span those obtained in the blood with anti-HIV nucleoside analogs and also recently reported by Painter and colleagues studying blood levels of rNHC in people dosed with molnupiravir [2]. In addition, using these concentrations we found a dose-response pattern for incorporation of mutations in viral RNA, inconsistent with drug precipitation during culture conditions. As to purity, it would be more relevant to repeat this experiment with clinical grade material, which we would be happy to do.

It is hard to argue that a ribonucleoside precursor to both RNA and DNA goes into one but not the other. Also, the known mutagenicity of hydroxylamine (which generates dNHC in DNA) suggests that if DNA repair could target such a small change in a base, it must do so in an incomplete way. This leads to the conclusion that treatment with molnupiravir will lead to mutations in host DNA in dividing cells. Using negative results to justify this risk as being unimportant is to create a blind spot for potential long term harm. Until a better understanding of treatment with molnupiravir is achieved, we would argue that its use should be limited to people with co-factor risks for COVID who are likely to receive the greatest benefit while being exposed to the unknown long term risks of exposure to this mutagen.

**Reference:**

1. Zhou S, Hill CS, Sarkar S, et al. beta-D-N 4-hydroxycytidine (NHC) Inhibits SARS-CoV-2 Through Lethal Mutagenesis But Is Also Mutagenic To Mammalian Cells. J Infect Dis **2021**.

2. Painter WP, Holman W, Bush JA, et al. Human Safety, Tolerability, and Pharmacokinetics of Molnupiravir, a Novel Broad-Spectrum Oral Antiviral Agent with Activity Against SARS-CoV-2. Antimicrob Agents Chemother **2021**:e02428-20.