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# Structure-activity relationship analysis of mitochondrial toxicity caused by antiviral ribonucleoside analogs

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Recent cases of severe toxicity during clinical trials have been associated with antiviral ribonucleoside analogs (e.g. INX-08189 and balapiravir). Some have hypothesized that the active metabolites of toxic ribonucleoside analogs, the triphosphate forms, inadvertently target human mitochondrial RNA polymerase (POLRMT), thus inhibiting mitochondrial RNA transcription and protein synthesis. Others have proposed that the prodrug moiety released from the ribonucleoside analogs might instead cause toxicity. Here, we report the mitochondrial effects of several clinically relevant and structurally diverse ribonucleoside analogs including NITD-008, T-705 (favipiravir), R1479 (parent nucleoside of balapiravir), PSI-7851 (sofosbuvir), and INX-08189 (BMS-986094). We found that efficient substrates and chain terminators of POLRMT, such as the nucleoside triphosphate forms of R1479, NITD-008, and INX-08189, are likely to cause mitochondrial toxicity in cells, while weaker chain terminators and inhibitors of POLRMT such as T-705 ribonucleoside triphosphate do not elicit strong *in vitro* mitochondrial effects. Within a fixed 3'-deoxy or 2′-*C*-methyl ribose scaffold, changing the base moiety of nucleotides did not strongly affect their inhibition constant ($K_i$) against POLRMT. By swapping the nucleoside and prodrug moieties of PSI-7851 and INX-08189, we demonstrated that the cell-based toxicity of INX-08189 is mainly caused by the nucleoside component of the molecule. Taken together, these results show that diverse 2′ or 4′ mono-substituted ribonucleoside scaffolds cause mitochondrial toxicity. Given the unpredictable structure-activity relationship of this ribonucleoside liability, we propose a rapid and systematic *in vitro* screen combining cell-based and biochemical assays to identify the early potential for mitochondrial toxicity.

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## 1. Introduction

Nucleoside analogs play an important role in the fight against viral diseases. Recently, the approval of sofosbuvir for the treatment of chronic hepatitis C virus (HCV) infection has opened the door to the development of ribonucleoside analogs against HCV and other RNA viruses (Herbst and Reddy, 2013; McQuaid et al., 2015). The main attributes of nucleoside analogs such as sofosbuvir are 1) a broad antiviral spectrum typically covering all strains of the same virus and 2) a high barrier to resistance (McCown et al., 2008). Unfortunately, a number of ribonucleoside analogs have failed at various stages of preclinical and clinical development due to safety and toxicity concerns. For example, valopicitabine (NM283, ester prodrug of 2′-*C*-Me-C) and balapiravir (R1626, ester prodrug of 4'-azido-C), two anti-HCV cytidine analogs, could not be advanced beyond phase 2 studies due to gastrointestinal and hematological toxicity, respectively (Brown, 2009; Nelson et al., 2012). The clinical development of INX-08189 (BMS-986094), a monophosphate prodrug of 2'-methyl-6-methoxy-guanosine intended for the treatment of chronic hepatitis C, was also halted due to severe cardiac toxicity that had not been previously identified through preclinical pharmacology profiling (Gentile et al., 2015). The anti-dengue 7-deaza -2'-ethynyl- -adenosine (NITD-008) also demonstrated safety concerns in animals that were not predicted from *in vitro* assessments (Yin et al., 2009). These examples show that ribonucleoside toxicity tends to be compound specific and therefore not associated with any particular nucleobase, sugar, or phosphate

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**Abbreviations**

| Abbreviation | Definition |
|---|---|
| NITD-008-TP | 7-deaza- 2′-*C*-ethynyl -adenosine triphosphate |
| INX-08189-TP | 2′-*C*-methyl-guanosine triphosphate |
| PSI-7851-TP | 2′-Fluoro-2′-*C*-methyl-uridine triphosphate |
| T-705 RTP | T-705 ribofuranosyl triphosphate |
| R1479-TP | 4′-azido-cytidine triphosphate |
| ATP | Adenosine triphosphate |
| GTP | Guanosine triphosphate |
| UTP | Uridine triphosphate |
| CTP | Cytidine triphosphate |
| 2′-*C*-Me-UTP | 2′-*C*-methyl uridine triphosphate |
| 2′-*C*-Me-CTP | 2′-*C*-methyl cytidine triphosphate |
| 3′-dATP | 3′-deoxy adenosine triphosphate |
| 3′-dGTP | 3′-deoxy guanosine triphosphate |
| 3′-dCTP | 3′-deoxy cytidine triphosphate |
| 3′-dUTP | 3′-deoxy uridine triphosphate |
| COX-I | cytochrome *c* oxidase I |
| DdRp | DNA-dependent RNA polymerase |
| DTT | Dithiothreitol |
| HCV | hepatitis C virus |
| POLRMT | human mitochondrial RNA polymerase |
| $K_i$ | inhibition constant |
| NTP | nucleoside triphosphate |
| SDH-A | succinate dehydrogenase A |
| TCA | trichloroacetic acid |

prodrug modification, making it difficult to predict in the early stages of medicinal chemistry programs. Furthermore, these examples highlight the need to develop new *in vitro* assays to assess the potential safety liability associated with ribonucleoside analogs.

Attempts to better understand and prevent toxicity of nucleoside analogs have often revealed a lack of selectivity against human polymerases, as exemplified with HIV therapeutics targeting DNA polymerase gamma (Bienstock and Copeland, 2004; Cote, 2005; Feng et al., 2001; Johnson et al., 2001). The ribonucleoside triphosphate forms of the HCV inhibitors balapiravir and INX-08189 are also known to be substrates of human mitochondrial RNA polymerase (POLRMT), blocking mitochondrial RNA transcription and protein synthesis (Arnold et al., 2012). These off-target enzyme-inhibitor interactions involving ribonucleotides were confirmed to be relatively specific to POLRMT and did not involve other human DNA or RNA polymerases except RNA polII (Feng et al., 2016). The unwanted POLRMT-specific targeting by balapiravir and INX-08189 might be explained by the lack of structural similarity between the POLRMT and other human polymerases, and the hypothesized unique bacteriophage origin of POLRMT (Ringel et al., 2011; Shutt and Gray, 2006). Importantly, balapiravir and INX-08189 were also associated with mitochondrial toxicity in cells, whereas sofosbuvir did not cause mitochondrial inhibition at the biochemical or cellular level (Arnold et al., 2012; Feng et al., 2016). This proposed mechanism involving POLRMT as the main off-target interaction has been used to explain the toxicity of other ribonucleoside analogs (Fenaux et al., 2016), but has also been contested by others. In particular, a direct link between the *in vitro* effects of INX-08189 on mitochondria and the cardiotoxicity observed in humans and animals has been difficult to establish (Ahmad et al., 2015; Baumgart et al., 2016; Feng et al., 2017; Gill et al., 2017). Instead, the prodrug moiety of INX-08189 may be responsible for the observed toxicity, a mechanism that does not involve POLRMT (Ehteshami et al., 2016). These separate and sometimes conflicting reports highlight the necessity to study the underlying root of toxicity of ribonucleoside analogs and to further de-risk mitochondrial toxicity in the early stages of antiviral drug development.

To better understand the structure-activity relationship responsible for mitochondrial toxicity, we evaluated a structurally diverse set of clinically relevant ribonucleoside analogs targeting different viruses (Fig. 1). The molecules include the previously characterized HCV inhibitors R1479, INX-08189, and PSI-7851; the anti-influenza drug T-705 (favipiravir) (Baranovich et al., 2013; Furuta et al., 2002, 2005, 2009, 2013, Oestereich et al., 2014); and the anti-dengue and anti-zika nucleoside analog, NITD-008 (Deng et al., 2016; Yin et al., 2009). The *in vitro* effect of each molecule on mitochondrial RNA synthesis was compared with the POLRMT interaction and inhibition profile of their corresponding triphosphate metabolites. In addition, we measured the effect of changing the base moiety of 3'-deoxy and 2′-*C*-methyl nucleotides on the POLRMT inhibition constant ($K_i$). Finally, we evaluated the potential impact of the monophosphate prodrug by swapping the nucleoside and prodrug moieties of PSI-7851 and INX-08189 and measuring the mitochondrial toxicity of the resulting hybrid molecules.

## 2. Materials and methods

### *2.1. Reagents*

Natural nucleoside triphosphates, ATP, CTP, GTP and UTP, were purchased from Trilink (San Diego, CA). Radiolabeled nucleoside triphosphates were purchased from PerkinElmer (Waltham, MA). All nucleoside analogs, their prodrugs and 5′-*O*-triphosphates used in this study were synthesized at Alios BioPharma, Inc (South San Francisco, CA) Tris-HCl buffer, NaCl and $MgCl_2$ solutions were purchased from Life Technologies. Trichloroacetic acid (TCA) (20% w/v) was purchased from BDH (Radnor, PA). Human mitochondrial RNA polymerase (POLRMT), human mitochondrial transcription factor A (TFA), human mitochondrial mitochondrial transcription factors B2 (TFB2) were purchased from Indigo Biosciences (State College, PA). Oligonucleotide pAA and pAAAGA were purchased from Dharmacon (Lafayette, CO).

### *2.2. Gel-based nucleotide incorporation by POLRMT using short DNA templates*

The oligonucleotides required for the POLRMT assay were custom synthesized at Dharmacon and contained the following sequence: (primer) 5′-UUUUGCCGCGCC-3′, (template 1) 3′-CGGCGCGG**TA**CG-TAAGGG-5′, and (template 2) 3′-CGGCGCGG**CA**CGTAAGGG-5′, (template 3) 3′-CGGCGCGG**GT**ACTAAGGG-5′, (template 4) 3′-CGGCGCGG**AT**CGTAAGG G-5’. The DNA-dependent RNA polymerase (DdRp) assay with POLRMT was performed under single-turnover conditions where enzyme concentration is in excess of the primer/template. Therefore, the $^{33}$P-RNA/DNA primer/template was used at 100 nM together with 320 nM enzyme. The standard 10-μL reactions were carried out at 30 °C for 1 min with 100 μM of each NTP, 10 mM $MgCl_2$, 50 mM NaCl, 40 mM Tris, pH 7.5, and 1 mM dithiothreitol (DTT). The reaction was stopped by adding 20 μL of formamide loading dye containing 50 mM EDTA. RNA products were resolved by electrophoresis on 22.5% TBE-Urea polyacrylamide sequencing gels. The radiolabeled band was quantified using a Typhoon PhosphorImager and ImageQuant 5.2 software.

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