Substrate analogs that trap the 2′-phospho-ADP-ribosylated RNA intermediate of the Tpt1 (tRNA 2′-phosphotransferase) reaction pathway

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ABSTRACT
The enzyme Tpt1 removes an internal RNA 2′-PO4 via a two-step reaction in which: (i) the 2′-PO4 attacks NAD+ to form an RNA-2′-phospho-(ADP-ribose) intermediate and nicotinamide; and (ii) transesterification of the ADP-ribose O2′ to the RNA 2′-phosphodiester yields 2′-OH RNA and ADP-ribose-1′,2′-cyclic phosphate. Because step 2 is much faster than step 1, the ADP-ribosylated RNA intermediate is virtually undetectable under normal circumstances. Here, by testing chemically modified nucleic acid substrates for activity with bacterial Tpt1 enzymes, we find that replacement of the ribose-2′-PO4 nucleotide with arabinose-2′-PO4 selectively slows step 2 of the reaction pathway and results in the transient accumulation of high levels of the reaction intermediate. We report that replacing the NMN ribose of NAD+ with 2′-fluoaroarabinose (thereby eliminating the ribose O2′ nucleophile) results in durable trapping of RNA-2′-phospho-(ADP-fluoaroarabinose) as a “dead-end” product of step 1. Tpt1 enzymes from diverse taxa differ in their capacity to use ara-2′F-NAD+ as a substrate.

Keywords: ADP-ribosylation; RNA 2′-phosphotransferase; nicotinamide adenine dinucleotide; transesterification

INTRODUCTION

tRNA 2′-phosphotransferase (Tpt1) is an essential enzyme in the fungal and plant tRNA splicing pathways that removes the 2′-PO4 at the splice junction generated by fungal and plant tRNA ligases (Culver et al. 1997). Tpt1 catalyzes a two-step reaction whereby: (i) the internal RNA 2′-PO4 attacks NAD+ to form an RNA-2′-phospho-(ADP-ribose) intermediate; and (ii) transesterification of the ribose O2′ to the 2′-phosphodiester yields 2′-OH RNA and ADP-ribose-1′,2′-cyclic phosphate (Supplemental Fig. S1; McCraith and Phizicky 1991; Culver et al. 1993; Spinelli et al. 1999; Steiger et al. 2005; Munir et al. 2018a). Whereas Tpt1 homologs with verified RNA 2′-phosphotransferase activity in vitro and in vivo are present in taxa other than fungi and plants (Spinelli et al. 1998; Sawaya et al. 2005; Munir et al. 2018a), their roles and their potential endogenous substrates in these taxa (bacteria, metazoa, and archaea) are unknown. Some Tpt1 orthologs catalyze additional NAD+-dependent ADP-ribosyltransferase reactions—such as ADP-ribose capping of RNA and DNA 5′-phosphate ends (Munir et al. 2018b) and removal of nucleic acid 2′ or 3′ terminal monophosphates (Munir et al. 2019)—albeit less vigorously than the canonical internal RNA 2′-phosphate removal reaction. Such findings raise interesting questions about Tpt1 substrate recognition and how it might vary among taxa.

Renewed interest in Tpt1 is prompted by its candidacy as a target for discovery of anti-fungal drugs, which is based on the fact that the enzymatic mechanism of tRNA splicing in metazoa—mediated by the RNA ligase RtcB—is entirely different from that of fungi and does not result in a junction 2′-PO4 (Popow et al. 2012). Whereas mammals do have a homolog of Tpt1, it plays no essential role in mammalian physiology, insofar as a homozygous tpt1-KO mouse is viable, develops normally, and has no defects in protein synthesis (Harding et al. 2008). This is in contrast to fungi, where Tpt1 is essential for viability...
in the model fungi Saccharomyces cerevisiae and Schizosaccharomyces pombe and in the pathogenic fungus Candida albicans (Segal et al. 2018).

One can envision two ways to interdict Tpt1 activity: (i) by inhibiting transfer of ADP-ribose from NAD\(^+\) to 2'-PO\(_4\) RNA, thereby resulting in the accumulation of spliced tRNAs that retain the 2'-PO\(_4\) in the anticodon loop; or (ii) by inhibiting the transesterification step and trapping the ADP-ribosylated RNA intermediate, thereby generating tRNAs with bulky lesions in the anticodon loop. Valuable insights into substrate recognition and the mechanism of the transesterification step emerged from a crystal structure of Clostridium thermocellum Tpt1 in a product-mimetic complex with ADP-ribose-1''-PO\(_4\) in the NAD\(^+\) site and pA\(_\text{p}\) in the RNA site (Banerjee et al. 2019) and from kinetic and mutational analyses of Runella slithyformis Tpt1 that identified a Arg–His–Arg–Arg catalytic tetrad in the active site and pinpointed one of the arginines as specifically essential for step 2 of the Tpt1 pathway (Munir et al. 2018a). Mutating this arginine slowed the step 1 rate by only threefold while slowing the step 2 rate by a factor of 214, thus resulting in the transient accumulation of very high levels of the normally evanescent ADP-ribosylated RNA intermediate.

Having created a situation in which the intermediate can be trapped by mutating the Tpt1 enzyme, we sought in the present study to achieve a similar outcome by chemical modifications of the NAD\(^+\) and 2'-PO\(_4\) RNA substrates. Our results highlight that replacement of the RNA ribose-2'-'PO\(_4\) nucleotide with arabinose-2'-PO\(_4\) and substitution of the NMN ribose of NAD\(^+\) with 2'-fluororibonucleoside result in transient and permanent trapping of the Tpt1 reaction intermediate, respectively.

RESULTS AND DISCUSSION

Utilization of ara-2''-fluoro NAD\(^+\) by Tpt1 enzymes traps the ADP-ribosylated RNA

The ara-2''F analog of NAD\(^+\) (shown in Fig. 1A) could, in principle, be able to support the first step in the Tpt1 pathway, but would be unable to undergo transesterification in the second step for lack of an O2' nucleophile. Because the effect of an arabinose sugar in lieu of ribose on the nicotinamide nucleoside moiety of NAD\(^+\) on substrate utilization by Tpt1 had not been queried previously, we surveyed four different Tpt1 enzymes (each at 0.5 µM concentration) for activity in the presence of 0.2 µM 5''-'PO\(_4\)-labeled 6-mer 2'-PO\(_4\)-branched RNA oligonucleotide (shown in Fig. 1B) and either 50 µM NAD\(^+\) or ara-2''F-NAD\(^+\). The Tpt1 proteins were from Runella slithyformis (a bacterium), Clostridium thermocellum (a bacterium), Chaetomium thermophillum (a fungus), and Homo sapiens (human). After a 30 min incubation at 37°C, the reaction products were resolved by urea-PAGE and visualized by autoradiography (Fig. 1A). Control reactions showed that each of the Tpt1 enzymes converted all of the input 2'-PO\(_4\) RNA substrate to a slower-migrating 2''-OH RNA product in the presence of 50 µM NAD\(^+\). When provided with 50 µM ara-2''F-NAD\(^+\), the Runella and Clostridium enzymes converted nearly all of the substrate into an even more slowly migrating species that corresponds to an ADP-ribosylated RNA (Munir et al. 2018a), which in this case is an RNA-2'-phospho-(ADP-fluororibosine) dead-end product of step 1 of the Tpt1 pathway (Fig. 1A). Chaetomium Tpt1 also formed the dead-end product in the presence of 50 µM ara-2''F-NAD\(^+\), though the extent of substrate conversion was lower. In contrast, human Tpt1 effected no detectable reaction of the 2'-PO\(_4\) RNA substrate in the presence of 50 µM ara-2''F-NAD\(^+\) (Fig. 1). We surmise that Tpt1 enzymes from different sources may vary in their sensitivity to the arabinose sugar modification of NAD\(^+\), but the utilization of ara-2''F-NAD\(^+\) as a substrate by Tpt1 does indeed result in trapping of the step 1 reaction product.

The temporal profiles of the reactions of Runella, Clostridium, and Chaetomium Tpt1 (0.5 µM) with 2'-PO\(_4\) RNA substrate (0.2 µM) and 50 µM ara-2''F-NAD\(^+\) are shown in Figure 1B. The data revealed a kinetic hierarchy whereby the apparent rate constant of the Clostridium enzyme for transfer of ADP-fluororibosine to the RNA 2'-phosphate (0.40 ± 0.022 min\(^-1\)) was fivefold faster than that of Runella Tpt1 (0.077 ± 0.0062 min\(^-1\)) and 16-fold faster than Chaetomium Tpt1 (0.025 ± 0.0028 min\(^-1\)). Although the yield of 2''-phospho-(ADP-fluororibosine) 6-mer RNA was high for Runella and Clostridium Tpt1, the apparent step 1 rate constant of Runella Tpt1 with 50 µM ara-2''F-NAD\(^+\) was at least two orders of magnitude slower than the step 1 rate constant observed with 1 mM NAD\(^+\) (Munir et al. 2018a). Clues to the slowing effect of the ara-2''F modification were gleaned by inspection of the crystal structure of Clostridium Tpt1 in complex with ADP-ribose-1''-PO\(_4\), a derivative of the ADP-ribose-1''-cyclic-PO\(_4\) reaction product that had undergone cyclic phosphate hydrolysis in situ (Fig. 1C; Banerjee et al. 2019). The ADP-ribose moiety occupies the NAD\(^+\) substrate site in the carboxy-terminal lobe of the Tpt1 tertiary structure and the 1''-PO\(_4\) moiety is the α anomer, consistent with stereochemical inversion at the nicotinamide ribose C1 of β-NAD\(^+\) during step 1 of the Tpt1 pathway. The ribose O2'' receives a hydrogen bond from Lys66, a residue conserved among Tpt1 family enzymes; Lys66 also makes a hydrogen bond to the 1''-PO\(_4\) (Fig. 1C). The ribose C2 atom makes van der Waals contact to the nearby Arg139 side chain (conserved among Tpt1 family enzymes) that engages the α and β phosphates of ADP-ribose/NAD\(^+\) (Fig. 1C). By superimposing a structure of ara-2''F (extracted from the ara-2''F-ADPR ligand in the 1.75 Å crystal structure of human CDC38; Liu et al. 2009) on the ribose of ADP-ribose-1''-PO\(_4\) in the Tpt1 complex, we
can see the “up” configuration of the ara-2′F moiety versus the “down” conformation of the ribose-2′OH (highlighted by the asterisk in Fig. 1C). In addition to eliminating the Lys66 contact to the NAD+ substrate, the ara-2′F modification introduces a steric clash between the 2′F atom and the Arg139 side chain. Note that the equivalent of Arg139 in *Runella* Tpt1 (Arg137) is critical for its RNA 2′-phospho-transferase activity in vitro and in vivo, as gauged by the effect of its replacement with alanine (Banerjee et al. 2019). We envision that the structural changes modeled in Figure 1C account, at least in part, for the slowing of the Tpt1 step 1 reaction with ara-2′F-NAD+.

**Runella** Tpt1 efficiently removes an internal 2′-phosphate from a DNA substrate

Previous studies of RNA repair enzymes that modify or join RNA ends have underscored the theme that their “RNA specificity” is dictated by a requirement for ribose at only a limited number of nucleotide positions within their
polynucleotide substrates, as gauged by the ability to replace many or most of the ribonucleotides with deoxyribonucleotides. For example, the RNA specificity of the RNA nick-sealing enzyme T4 RNA ligase 2 arises from a requirement for at least two ribonucleotides immediately flanking the 3′-OH of the nick; the rest of the nicked duplex can be replaced by DNA (Nandakumar and Shuman 2004). In the case of the plant tRNA ligase AtRNL, which acts via the same chemical pathway of healing and sealing as fungal tRNA ligase Trl1, a single ribonucleoside-2′,3′-cyclic-PO₄ moiety enables AtRNL to efficiently splice an otherwise inappropriate branched ribonucleotide (Chan et al. 2009). CthHen1 is an RNA ribose-2′-O-methyltransferase that marks the 3′ terminal nucleoside of broken RNAs and protects ligated repair junctions from iterative damage by transesterifying endonucleases (Chan et al. 2009). CthHen1 is adept at methylating a polynucleotide composed of 23 deoxyribonucleotides and one 3′ terminal ribonucleotide, signifying that it has no strict RNA specificity beyond the 3′ nucleoside (Jain and Shuman 2011).

To query the extent of the RNA requirement for Tpt1, we synthesized and then 5′-32P-labeled an analog of the 6-mer 2′-PO₄ oligonucleotide substrate in which the five nucleotides flanking the 2′-PO₄ branch nucleotide were replaced by deoxynucleotides (Fig. 2). The 2′-PO₄ RNA and DNA substrates (at 0.2 µM concentration) were reacted for 30 min with increasing amounts of Runella Tpt1 in the presence of 1 mM NAD⁺. The product distributions were quantified and are plotted as a function of input RslTpt1 (Fig. 2A,B). The salient points were that Runella Tpt1 is virtually equally adept at removing an internal 2′-PO₄ from RNA and DNA polynucleotides, without accumulating significant amounts of the 2′-phospho-ADP-ribosylated intermediate at limiting enzyme concentrations.

**Effect of 2′F-arabinose sugars flanking the 2′-phosphate branchpoint**

Arabino nucleic acid (ANA) is the 2′-epimer of RNA in which the chirality of the 2′ position of the pentose sugar is switched so that the 2′-OH group points up, that is, in the direction opposite that of the 2′ hydroxyl of a ribose. 2′-Fluoroarabino nucleic acid (2′-FANA) is the 2′-F analog of ANA, in which the sugar pucker mimics that of DNA (Martín-Pintado et al. 2012). We tested Runella Tpt1 activity on a 2′-FANA version of the 6-mer 2′-PO₄ oligonucleotide substrate in which the five nucleotides flanking the 2′-PO₄ branched ribonucleotide were replaced by 2′-fluoro-arabino nucleotides. RslTpt1 removed the 2′-PO₄ from the 2′-FANA substrate, albeit with approximately sevenfold lower specific activity compared to the 6-mer 2′-PO₄ RNA control substrate (Fig. 3A,B). The 2′-phospho-ADPribosylated intermediate accumulated to an extent of 4% of total labeled RNA at low enzyme concentrations (Fig. 3B). Thus, RslTpt1 activity is tolerant of DNA in lieu of RNA adjacent to the 2′-PO₄ branch but is less tolerant of a 2′-FANA nucleic acid.

**Effect of 3′–5′ phosphorothioates flanking the 2′-phosphate branchpoint**

Phosphorothioate (PS) modifications of the 3′–5′ phosphodiester backbone of DNA and 2′-FANA oligonucleotides provide stability against nuclease digestion while allowing for RNase H-mediated cleavage of complementary RNAs (Damha et al. 1998; Wilds and Damha 2000). As such, PS-DNA and PS-2′-FANA oligos are effective tools for antisense interference manipulations of gene expression in vivo (Kalota et al. 2006). Here we introduced racemic PS modifications at all five 3′–5′ phosphodiester

**FIGURE 2.** Runella Tpt1 efficiently removes an internal DNA 2′-phosphate. Reaction mixtures (10 µL) containing 100 mM Tris-HCl, pH 7.5, 1 mM NAD⁺, 0.2 µM (2 pmol) 5′-32P-labeled 6-mer 2′-PO₄ RNA (A), DNA (B, with deoxyribonucleotides in italics), or PS-DNA (C, with deoxyribonucleotides in italics) substrates, and Runella Tpt1 as specified were incubated at 37°C for 30 min. The reaction products were analyzed by urea-PAGE. The extents of formation of the 2′-OH product and the ADP-ribosylated intermediate are plotted as a function of input RslTpt1. Each datum is the average of three independent titration experiments ±SEM.
6-mer 2′-PO₄ DNA and 2′-FANA substrates (creating in each case a mixture of 32 PS isomers) and tested them for activity with Runella Tpt1. These PS-modified 2′-PO₄ oligos could be completely converted to 2′-OH products (Figs. 2C, 3C). However, the enzyme titration curves were shifted significantly to the right vis-à-vis the DNA and 2′-FANA substrates with unmodified phosphodiester backbones (Figs. 2B, 3B). Runella Tpt1 specific activity was reduced by 11-fold and sevenfold, respectively, by the PS modifications of DNA and 2′-FANA substrates. Moreover, there was substantial accumulation of the 2′-phospho-ADP-ribosylated intermediate at sub-saturating enzyme levels, to an extent of 27% and 30% of total nucleic acid for the PS-DNA and PS-2′-FANA substrates, respectively (Figs. 2C, 3C).

Effect of an arabinose sugar at the 2′-phosphate branchpoint

We synthesized a 6-mer RNA with an internal arabinose-2′-phosphate moiety (shown in Fig. 4D). A 30 min reaction of 0.2 μM (2 pmol) 5′-32P-labeled ara-2′-PO₄ RNA with increasing concentrations of Runella Tpt1 in the presence of 1 mM NAD⁺ resulted in the initial accumulation of very high levels of 2′-phospho-ADP-ribosylated intermediate, to a peak level of 70% of total RNA at 0.05 to 0.1 μM RslTpt1 (0.5 to 1 pmol), followed by its progressive conversion to 2′-OH product as enzyme concentration was increased up to 1 μM (10 pmol) (Fig. 4A). A kinetic analysis of the reaction under conditions of enzyme excess (0.2 μM ara-2′-PO₄ RNA; 1 μM RslTpt1) is shown in Figure 4B and revealed a precursor-product relationship whereby the ADP-ribosylated RNA intermediate accumulated steadily at early times and comprised 61% of total RNA at 1.5 min before declining steadily thereafter as all of the RNA was converted to 2′-OH product. The data fit well by nonlinear regression in Prism to a unidirectional two-step mechanism with apparent step 1 and step 2 rate constants of 1.50 ± 0.07 min⁻¹ and 0.35 ± 0.01 min⁻¹, respectively. Comparison of these data to reaction rates of Runella Tpt1 with the 6-mer ribose-2′-PO₄ RNA (step 1 and step 2 rate constants of 21 min⁻¹ and 221 min⁻¹, respectively; Munir et al. 2018a) highlights the profound impact of an arabinose sugar at the 2′-PO₄ branchpoint, which slows step 1 by a factor of 14 but exerts a much stronger 630-fold effect on step 2.

Because we do not yet have a structure of a Tpt1 enzyme in complex with an RNA containing a 2′-phosphate, we are unable to convincingly model how the arabinose-2′-PO₄ modification affects substrate binding and reaction chemistry. However, we can guess from the available Tpt1 structure with product-mimetic ligands that the extensive network of electrostatic interactions of essential Tpt1 amino acid side chains with the transferred phosphate moiety (Banerjee et al. 2019) would be perturbed if the 2′-phosphate were oriented “up” from the arabinose sugar instead of down from ribose. We predict this would misalign the 2′-phosphate nucleophile with respect to the ribose C1′ atom of NAD⁺ and the departing nicotinamide during step 1 catalysis. From the rate effects, we suspect that an “up” conformation of the arabinose-2′-phosphodiester to ADP-ribose even more severely misaligns the geometry of the ADP-ribose O2′ nucleophile and the RNA arabinose O2′ leaving group during step 2 catalysis.

Additive effect of an arabinose-2′-phosphate and the Runella Tpt1 R64A mutation

Runella Tpt1 amino acids Arg16, His17, Arg64, and Arg119 are conserved in all Tpt1 homologs and essential
Kinetic analysis of Runella Tpt1 alanine mutants implicated Arg64 and Arg16 as catalysts of the transesterification step. The Clostridium Tpt1 structure showed that the equivalent two arginines make bidentate contacts to the transferred 2′-PO₄ in the ADP-ribose-1′′-PO₄ product ligand, consistent with the arginines stabilizing the transition state of the transesterification step. Whereas R16A mutation of Runella Tpt1 also profoundly affects step 1 of the pathway, the R64A mutation is highly selective in its effect on step 2, leading to transient accumulation of very high levels of the ADP-ribosylated intermediate (Munir et al. 2018a). Because the R64A effect resembles that of the arabinose-2′-PO₄ modification, we tested the reaction of the R64A mutant with the ara-2′-PO₄ RNA substrate (Fig. 4C). We found that RslTpt1-R64A catalyzed quantitative conversion of the ara-2′-PO₄ substrate to 2′-phospho-ADP-ribosylated RNA over a 30 min time course with an apparent rate constant of 0.28 ± 0.013 min⁻¹. There was no detectable formation of a 2′-OH product. The rate constant of the R64A mutant for transfer of ADP-ribose to the arabinose-2′-phosphate was fivefold slower than that of wild-type Runella Tpt1. The effect of the R64A mutation on step 1 catalysis at the arabinose-2′-phosphate was similar to the threefold rate decrement in step 1 catalysis by the R64A mutant at a ribose-2′-phosphate (Munir et al. 2018a).

**Effect of an arabinose-2′-phosphate on Clostridium Tpt1**

To see if the intermediate-trapping effect of the ara-2′-PO₄ modification extends to another Tpt1 ortholog, we performed a 30 min reaction of 0.2 µM (2 pmol) 5′-³²P-labeled ara-2′-PO₄ RNA and 1 mM NAD⁺ with increasing concentrations of Clostridium thermocellum Tpt1. This resulted in an initial accumulation of 2′-phospho-ADP-ribosylated intermediate, to a peak level of 65% of total RNA at 0.1 µM CthTpt1 (1 pmol), followed by its progressive

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**FIGURE 4.** Effect of an arabinose sugar at the 2′-phosphate branchpoint. (A) Reaction mixtures (10 µL) containing 100 mM Tris-HCl, pH 7.5, 1 mM NAD⁺, 0.2 µM (2 pmol) 5′-³²P-labeled 6-mer ara-2′-PO₄ substrate (shown in D), and 0, 0.25, 0.5, 0.75, 1, 2.5, 5, 7.5, or 10 pmol wild-type RslTpt1 were incubated at 37°C for 30 min. (B, C) Reaction mixtures (100 µL) containing 100 mM Tris-HCl, pH 7.5, 1 mM NAD⁺, 0.2 µM 5′-³²P-labeled 6-mer ara-2′-PO₄ substrate, and 1 µM wild-type RslTpt1 (B) or RslTpt1-R64A (C) were incubated at 37°C. The reactions were initiated by adding enzyme to a prewarmed reaction mixture. Aliquots (10 µL, containing 2 pmol of RNA) were withdrawn at the times specified and quenched immediately with 3 volumes of cold 90% formamide, 50 mM EDTA. The reaction products were analyzed by urea-PAGE and quantified by scanning the gels. The extents of formation of the 2′-OH product and the ADP-ribosylated intermediate are plotted as a function of input RslTpt1 (panel A) or reaction time (panels B, C). Each datum is the average of three independent titration or time-course experiments (±SEM). (D) Chemical structure of the arabinose-2′-PO₄ branchpoint.
conversion to 2'-OH RNA product as enzyme concentration was increased up to 1 µM (10 pmol), at which point the intermediate and product comprised 12% and 88% of total RNA, respectively (Fig. 5A). The residual intermediate was not diminished when the input enzyme was increased to 0.3 µM (30 pmol) (Fig. 5A). A kinetic analysis of the reaction in enzyme excess (0.2 µM ara-2'-PO₄ RNA; 1 µM CthTpt1) showed accumulation of the intermediate at early times to an extent of 85% of total RNA at 1.5 min before its conversion thereafter to 2'-OH product (Fig. 5B). The apparent step 1 and step 2 rate constants were 2.19 ± 0.16 min⁻¹ and 0.060 ± 0.0018 min⁻¹, respectively (Fig. 5B). Thus, the rate of step 2 transesterification at an arabinose-2'-PO₄ was 37-fold slower than the rate of step 1 ADP-ribosylation.

Conclusions
The present study illuminates the distinctive effects of non-ribose sugars in the 2'-PO₄ nucleic acid and NAD⁺ substrates on Tpt1 activity. Whereas replacement of each of the ribose sugars flanking the internal 2'-PO₄ with a deoxynucleotide did not affect the efficiency of 2'-PO₄ removal by Runella Tpt1, their replacement with 2'-fluoroarabinose reduced Tpt1 specific activity, albeit without accumulation of the 2'-phospho-ADP-ribosylated intermediate. In contrast, replacing the ribose of the 2'-PO₄ nucleotide with arabinose selectively and severely reduced the rate of the transesterification step and thereby resulted in the build-up of very high levels of the reaction intermediate during catalysis by Runella and Clostridium Tpt1. Replacing the NMN ribose of NAD⁺ with 2'-fluoroarabinose (which eliminates the step 2 ADP-ribose O₂⁻⁻ nucleophile) results in trapping of RNA-2'-phospho-(ADP-fluoroarabinose) as a dead-end step 1 product. We find that Tpt1 orthologs differ in their ability to use ara-2'-F-NAD⁺.

Our identification of substrate analogs that trap the ADP-ribosylated intermediate has implications for the development of Tpt1 “poisons” as anti-fungals. A poison elicits the formation of a reaction intermediate that is potentially more deleterious (in this case by virtue of the large bulk of 2'-phospho-ADP ribose at the splice junction compared to the 2'-phosphate) than an inhibitor that results in the mere accumulation of 2'-phosphate substrate. Indeed, DNA topoisomerase poisons that trap covalent topoisomerase-DNA intermediates are among the most successful and widely used anti-bacterial and anti-cancer agents in clinical practice (Pommier 2013).

MATERIALS AND METHODS

Recombinant Tpt1 proteins
Tpt1 enzymes from Clostridium thermocellum, Homo sapiens, Runella sillyformis, and Chaetomium thermophilum were produced in E. coli and purified as described previously (Munir et al. 2018a,b).

Solid-phase synthesis of oligonucleotides with internal 2'-phosphates

General methods
Oligonucleotide syntheses were carried out using an ABI 3400 DNA synthesizer (Applied Biosystems) on a Unylinker (ChemGenes) solid support at a 1 µmol scale. Conventional

FIGURE 5. Effect of an arabinose-2'-PO₄ on Clostridium Tpt1. (A) Reaction mixtures (10 µL) containing 100 mM Tris-HCl, pH 7.5, 1 mM NAD⁺, 0.2 µM (2 pmol) 5'-32P-labeled 6-mer ara-2'-PO₄ substrate, and 0, 0.5, 1, 2.5, 5, 10, or 30 pmol Clostridium thermocellum (Cth) Tpt1 were incubated at 37°C for 30 min. (B) A reaction mixture (100 µL) containing 100 mM Tris-HCl, pH 7.5, 1 mM NAD⁺, 0.2 µM 5'-32P-labeled 6-mer ara-2'-PO₄ substrate, and 1 µM wild-type CthTpt1 was incubated at 37°C. The reaction was initiated by adding enzyme to a prewarmed reaction mixture. Aliquots (10 µL, containing 2 pmol of RNA) were withdrawn at the times specified and quenched immediately with 3 volumes of cold 90% formamide, 50 mM EDTA. The reaction products were analyzed by urea-PAGE and quantified by scanning the gels. The extents of formation of the 2'-OH product and the ADP-ribosylated intermediate are plotted as a function of input CthTpt1 (panel A) or reaction time (panel B). Each datum is the average of three independent titration or time-course experiments (±SEM).
deoxyribonucleoside, 2′-tert-butyl-dimethylsilyl (TBDMs) ribonucleoside, 2′-fluoro-arabinonucleoside (2′-FANA) and 2′-acetyl levulinyl (ALE) ribonucleoside phosphoramidites (0.15 M in MeCN) (ChemGenes) were used. Additionally, 2′-arabinono levulinyl (Lev) phosphoramidite was synthesized as described previously (Katolik et al. 2014). For phosphorylation, bis-cyanoethyl-\( -N\), N-diisopropyl phosphoramidite (0.20 M in MeCN) was used. Phosphoramidites were dissolved in MeCN and activated with 5-ethylthio-1H-tetrazole (0.25 M in MeCN). Capping was carried out by the simultaneous delivery of acetic anhydride in pyridine/5-ethylthio-1H-tetrazole (0.25 M in MeCN). Synthesis of RNA sequences containing 2′-phosphate-containing oligonucleotides was effected with 0.1 M iodine in pyridine/H\(_2\)O/THF (20 sec); a solution of 3% trichloroacetic acid in THF, delivered over 1.8 min, was used to deprotect DMTr groups. For 2′-phosphate-containing substrates, a solution of anhydrous TEA/MeCN (2:3 v/v) was used to remove cyanoethyl phosphate protecting groups, while a 0.5 M solution of hydrazine hydrate in pyridine/\( \text{AcOH} \) (3:2 v/v) was used to remove ALE protecting groups. All oligonucleotides were deprotected and cleaved from the solid support using an ammonium hydroxide and ethanol solution. TBDMS groups were removed using TREAT-HF. Crude oligonucleotides were purified via HPLC and characterized by LC–MS.

**Synthesis of 2′ phosphate-containing oligonucleotides**

Synthesis of RNA sequences containing 2′-phosphate moieties was carried out as previously described (Murir et al. 2018a). The equivalent sequences containing DNA or 2′-FANA at positions flanking the 2′-phosphate RNA branchpoint were also synthesized in this manner using DNA or 2′-FANA phosphoramidites, respectively. For the arabinose-2′-phosphate (ANA 2′-PO\(_4\))-containing sequence: 5′-\( \text{CrCrA} \)AraA(2′-ALE groups)-P)rArU-3′, the first section of the oligonucleotide: 5′-araA(2′-Lev)-O-Lev-3′, was synthesized on the solid support in the conventional 3′ to 5′ solid phase synthesis. The araA(2′-Lev) unit in this sequence was introduced by coupling a 5′-DMTr-2′-O-Lev-3′-OCE arabinose phosphoramidite (0.15 M in MeCN) for 15 min. In the case of arabinonucleosides, the removal of the backbone cyanoethyl phosphodiester protecting groups is not necessary, since the trans orientation of the free 2′-OH to the 3′-phosphate triester will prevent chain cleavage at the arabinose position. To remove the 2′-ALE groups, the columns were returned to the synthesizer and a freshly prepared solution of 0.5 M hydrazine hydrate in pyridine/\( \text{AcOH} \) (3:2 v/v) was flowed through the columns (20 sec flow + 3.75 min sleep, repeated 4×). After washing (MeCN, 10 min) and drying (Ar gas, 10 min), the solid supports were dried again in vacuo (30 min). To phosphitylate at the newly exposed 2′-OH, bis-cyanoethyl-\( -N\), N-diisopropyl CED phosphoramidite (0.20 M in MeCN) was coupled for 30 min, and then further oxidized using 0.1 M iodine in pyridine/H\(_2\)O/THF (20 sec). To complete the oligonucleotide, standard 3′ to 5′ synthesis was continued on the 5′ terminus of the growing oligonucleotide using the sequence 5′rCrCrA-3′ to yield the desired hexamer oligonucleotide substrate.

Deprotection and cleavage of oligonucleotides from the solid support was achieved by treatment with 1 mL of cold 29% aqueous ammonia/ethanol (3:1 v/v) for RNA and 2′-FANA oligonucleotides, and 1 mL of cold 29% aqueous ammonia for DNA oligonucleotides for 16 h at 65°C. Samples were centrifuged and the supernatant was transferred to a clean 1.5 mL eppendorf tube and vented for 30 min, chilled on dry ice, and evaporated to dryness. Removal of the 2′-silyl protecting groups for the RNA oligonucleotides was achieved by treatment with a 300 µL solution of NMP/\( \text{Et}_{4} \)N/TREAT-HF (3:4:6 v/v/v) for 90 min at 65°C, followed by quenching with 3 M NaOAc buffer (50 µL; pH 5.5) and precipitation of the crude oligonucleotide from cold butanol (1 mL, −20°C). Samples were chilled on dry ice for 30 min and then centrifuged. After removing the supernatant, the remaining pellet (containing oligonucleotide) was evaporated to dryness, taken up in autoclaved milliQ water (1 mL), filtered, and quantified by UV spectroscopy.

Crude oligonucleotides were HPLC-purified using a Waters Protein-Pak DEAE 5PW anion exchange column (21.5 × 150 mm). A mobile phase of 1 M aqueous LiClO\(_4\) in milliQ water was used for analysis and purification (0%–20% LiClO\(_4\) over 30 min, 4 mL/min, 60°C). Following collection of the desired peaks, fractions were combined and excess LiClO\(_4\) salts were removed using Gel Pak 2.5 size exclusion columns (Glen Research). Purified oligonucleotides were characterized by electrospray ionization-mass spectrometry (the HPLC elution profiles and MS analyses are shown in Supplemental Figs. S2–S4) and quantified by UV spectroscopy. Extinction coefficients were determined using the IDT OligoAnalyzer tool (www.idtdna.com/analyzer/Applications/OligoAnalyzer). The oligonucleotides were stored at −20°C.

**5′ \( {\text{32P}} \)-labeled oligonucleotide substrates**

Synthetic 6-mer oligonucleotides 5′-CCAA \( ^{32P} \)AU containing an internal 2′-PO\(_4\) were 5′-\( ^{32P} \)-labeled by reaction with phosphatase-dead T4 polynucleotide kinase (Phik-D167N) in the presence of \([\text{\(^{32P}\)}]\text{ATP}\). The reactions were quenched with 90% formamide, 50 mM EDTA, 0.01% xylene cyanol and the radioactively labeled RNAs were purified by electrophoresis through a 40-cm 20% polyacrylamide gel containing 7 M urea in 45 mM Tris-borate, 1 mM EDTA. The radioactively labeled oligonucleotides were eluted from excised gel slices, recovered by ethanol precipitation, and resuspended in 10 mM Tris-HCl, pH 6.8, 1 mM EDTA, and stored at −20°C.

**Assay of Tpt1 activity**

Reaction mixtures containing 100 mM Tris-HCl (pH 7.5), 0.2 µM 5′-\( ^{32P} \)-labeled nucleic acid substrates, NAD\(^+\) or ara-2′-F-NAD\(^+\) as specified, and Tpt1 as specified in the figure legends were incubated at 37°C. The reactions were quenched at the times specified in the figure legends by addition of 3 volumes of cold 90% formamide, 50 mM EDTA. The products were analyzed by electrophoresis (at 55 W constant power) through a 40-cm 20% polyacrylamide gel containing 7 M urea in 45 mM Tris-borate, 1 mM EDTA and visualized by autoradiography and/or scanning the gel with a Fujifilm FLA-7000 imaging device. The products were quantified by analysis of the gel scans in ImageQuant. Ara-2′-F-NAD\(^+\) was purchased from BIOLOG (Bremen, Germany; Cat. No. D148).

**SUPPLEMENTAL MATERIAL**

Supplemental material is available for this article.
ACKNOWLEDGMENTS

This work was supported by grants from the U.S. National Institutes of Health, National Institute of General Medical Sciences (R35-GM126945) and the National Science and Engineering Research Council of Canada (Discovery grant to M.J.D.).

Received December 17, 2019; accepted January 10, 2020.

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