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Evaluating TNA stability under simulated physiological conditions

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ABSTRACT

Chemically modified oligonucleotides are routinely used as diagnostic and therapeutic agents due to their enhanced biological stability relative to natural DNA and RNA. Here, we examine the biological stability of α-L-threofuranosyl nucleic acid (TNA), an artificial genetic polymer composed of repeating units of α-L-threofuranosyl sugars linked by 2′,3′-phosphodiester bonds. We show that TNA remains undigested after 7 days of incubation in the presence of either 50% human serum or human liver microsomes and is stable against snake venom phosphordiesterase (a highly active 3′ exonuclease). We further show that TNA will protect internal DNA residues from nuclease digestion and shield complementary RNA strands from RNA degrading enzymes. Together, these results demonstrate that TNA is an RNA analogue with high biological stability.

Chemically modified oligonucleotides are valuable tools in the development of oligonucleotide-based diagnostic and therapeutic applications.[1–5] To date, hundreds of modifications have been made to enhance the stability of therapeutic ribozymes, aptamers, small interfering RNAs (siRNAs) and antisense oligonucleotides (AO). From this work, structure–activity relationships have begun to emerge that correlate hybridization efficiency with changes in oligonucleotide structure.[6] This knowledge is now being used to examine the phenotypic effects of RNA-based therapeutics on biological targets.[7–9] While most studies have focused on subtle chemical changes made to the ribose sugar and phosphodiester linkage, recent advances in nucleic acid chemistry have led to the emergence of xeno-nucleic acid (XNA) polymers with backbone structures that are distinct from those found in nature.[10,11] XNAs with diverse backbone structures represent a promising new category of therapeutic oligonucleotides, as their structures are expected to be more resistant to nuclease degradation than DNA and RNA (and close structural analogues thereof).[12]

Herein, we examine the biological stability of TNA (α-L-threofuranosyl-(3′,2′) nucleic acid, Fig. 1), which is an artificial genetic polymer in which the natural ribose sugar found in RNA has been replaced by a four-carbon α-L-threofuranose sugar.[13] In contrast to DNA and RNA, which have a six-atom backbone repeat unit connected by 3′,5′-phosphodiester linkages, TNA has a backbone

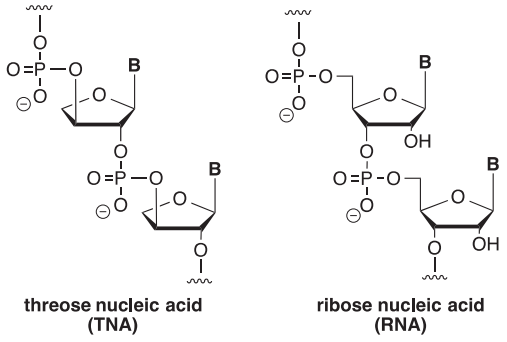

Figure 1. Constitutional structures for the linearized backbone of TNA and DNA. TNA contains one less atom per backbone repeat unit than DNA.

periodicity of five atoms with phosphodiester linkages occurring at the 2′ and 3′ positions of the furanose ring. The NMR structure of a self-complementary duplex reveals that TNA adopts an A-like helical geometry, which is consistent with its ability to form stable antiparallel Watson–Crick duplexes with complementary strands of DNA and RNA.[14] The ability to transfer genetic information between TNA and RNA, coupled with the chemical simplicity of threose relative to ribose, has fueled interest in TNA as a possible progenitor of RNA.[15] TNA is also being explored as a source of nuclease resistant affinity reagents (aptamers) and catalysts for synthetic biology and molecular medicine.[16] Recent advances in

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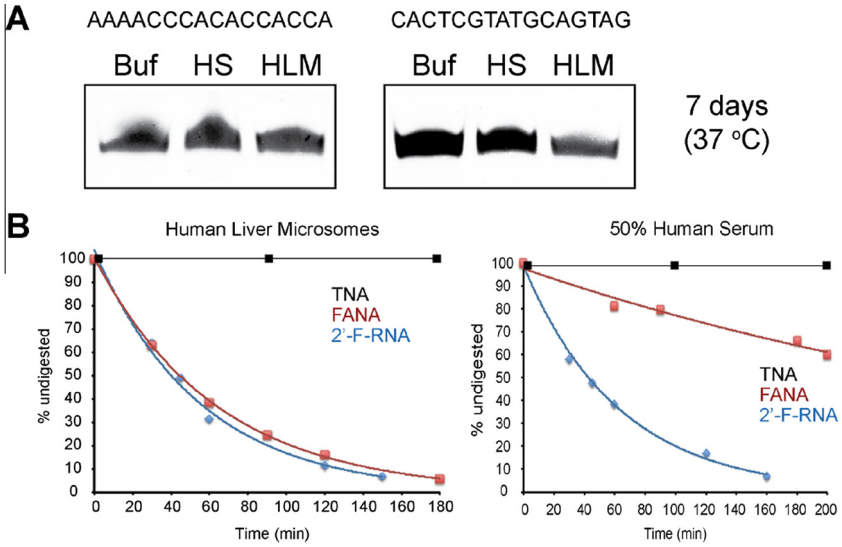

Figure 2. Comparative nuclease stability assays performed in human serum (HS) and human liver microsomes (HLM). (A) TNA polymers remain undigested after 7 days of incubation at 37 °C in 50% human serum or 0.5 mg/mL human liver microsomes. Buf denotes buffer only. (B) FANA and 2′-F-RNA both rapidly degrade under simulated biological conditions.

this area have led to the development of engineered polymerases that can copy genetic information back and forth between TNA and DNA.[17]

Previously, we have shown that TNA is resistant to nuclease digestion by recombinant DNase and RNase.[18] This observation motivated us to analyze the biological stability of TNA under simulated physiological conditions. In particular, we chose to examine the stability of TNA in the presence of human serum and human liver microsomes—two biologically relevant matrices commonly used to predict oligonucleotide stability in vivo.[19]

We began by comparing the stability of two different TNA strands in human serum and human liver microsomes. Both TNA oligonucleotides, as well as all subsequent TNA molecules used in this study, were constructed by solid-phase synthesis using chemically synthesized phosphoramidites. The TNA strands were incubated with either 50% human serum in DMEM media or 0.5 mg/mL human liver microsomes in reaction buffer for 7 days at 37 °C. The latter is a rigorous test of oligonucleotide stability, as the nuclease activity in microsomes is much stronger than human serum due to the high concentration and abundance of nucleases with different activities.[19] Following incubation, the TNA strands were evaluated by denaturing polyacrylamide gel electrophoresis (PAGE). As illustrated in Figure 2**A**, both TNA strands remain undigested after 7 days of incubation. This result was highly reproducible, indicating that the constitutional structure of TNA is highly resistant to biological degradation.

As a measure of comparison, we performed a time course analysis on 2′-deoxy-2′-fluoro-β-D-arabinonucleic acid (FANA) and 2′-deoxy-2′-fluoro-ribonucleic acid (2′-F-RNA), which are commonly used analogues in RNA therapeutics. When tested under identical conditions, both FANA and 2′-F-RNA degrade with a half-life ($T_{1/2}$) of ~45 min in 0.5 mg/mL human liver microsomes. In 50% human serum, FANA is more stable than 2′-F-RNA, but still shows significant degradation relative to TNA. In this case, 2′-F-RNA degrades with a $T_{1/2}$ of ~45 min, while FANA is ~40% digested after 200 min. The results observed in human serum are

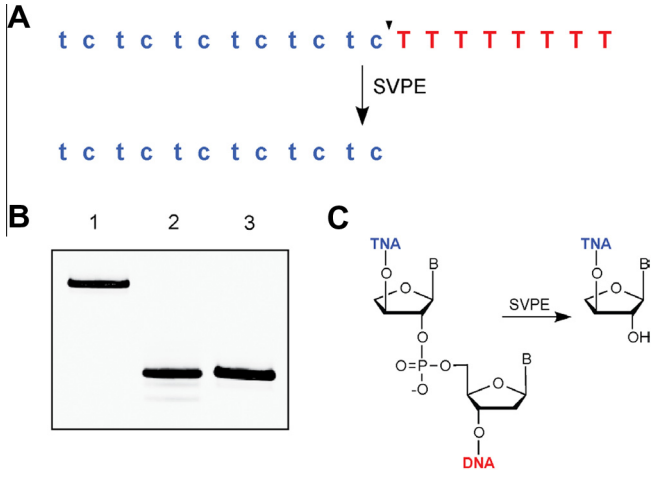

Figure 3. Stability of TNA towards snake venom phosphodiesterase. A. Schematic illustration of a chimeric TNA–DNA oligonucleotide before and after digestion with snake venom phosphodiesterase. TNA lower case blue letters. DNA upper case red letters. (b) Analysis of the reaction products by polyacrylamide gel electrophoresis. Lanes 1 and 2: Fam-labeled $(tc)_6$-dT_8 before and after treatment with SVPDE, respectively. Lane 3: size matched TNA product. (c) The cleavage product of $(tc)_6$-dT_8 after treatment with SVPDE as verified by ESI-TOF mass spectrometry.

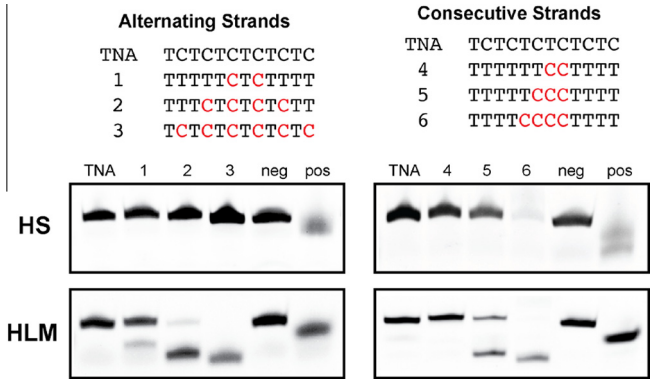

Figure 4. Nuclease stability of internal DNA residues. TNA oligonucleotides containing alternating and consecutive patterns of DNA residues show varying levels of nuclease stability in 50% human serum (HS) and 0.5 mg/mL human liver microsomes (HLM) after 24 h of incubation at 37 °C. An all DNA 2′d-C_{12} sequence (D1) was used for the negative (neg) and positive (pos) control for nuclease activity in the presence of HS or HLM, respectively.

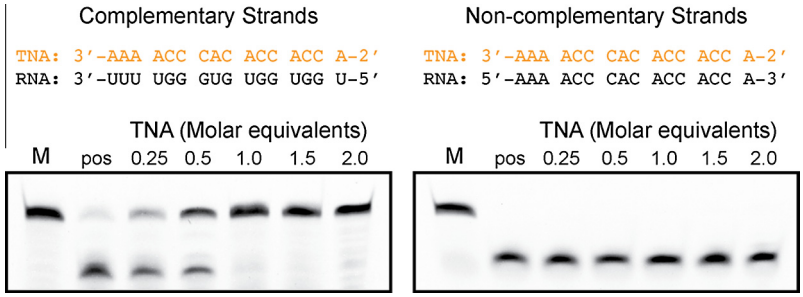

Figure 5. RNA protection assay. Complementary TNA protects RNA from degrading in the presence of 0.5 mg/mL human liver microsomes at 37 °C. M refers to the RNA marker in buffer only. A positive control (pos) indicates the level of RNA digestion in the absence of TNA. TNA amounts ranged from 0.2 to 2 molar equivalents.

consistent with previous literature values for the stability of FANA and 2′-F-RNA.[20,21] To our knowledge, FANA and 2′-F-RNA have not been examined previously in human liver microsomes.

Next, we examined the stability of TNA against snake venom phosphodiesterase (SVPDE), which is an aggressive enzyme with strong 3′-exonuclease activity. SVPDE is often used to evaluate oligonucleotide stability due to its strong degradative properties. As illustrated in Figure 3, a chimeric TNA–DNA oligonucleotide containing a DNA tail composed of 8 thymidine residues was treated with SVPDE for 24 h at 37 °C. Analysis of the SVPDE treated strand by denaturing PAGE revealed a single band that migrated with the same electrophoretic mobility as a size-matched product. ESI-TOF mass spectrometry (supplementary material) confirmed that SVPDE treatment produced the desired TNA molecule with a free 2′ hydroxyl group, indicating that cleavage occurred between the 2′ hydroxyl moiety of the threose ring and 5′-monophosphate of DNA. Efficient cleavage of the 2′,5′-TNA/DNA junction demonstrates that TNA is highly resistant to SVPDE treatment, which could prove useful in future TNA applications.

Given the high biological stability of TNA against nuclease degradation, we wished to examine the ability for TNA to protect internal DNA residues from enzymatic degradation. For this assay, we constructed a series of mosaic oligonucleotides with a mixed TNA–DNA backbone structure. The mosaic strands were synthesized with an increasing number of alternating or consecutive DNA residues to identify the maximum number of DNA residues that can be incorporated into a TNA strand while still maintaining nuclease resistance. The strands, along with all-TNA and all-DNA controls, were incubated with 50% human serum or 0.5 mg/mL human liver microsomes for 24 h at 37 °C. Denaturing PAGE analysis revealed different patterns of stability depending on whether the DNA residues were alternating or consecutive (Fig. 4). In human serum, all of the alternating DNA residues are protected, while the consecutive series is limited to 3 DNA residues. In human liver microsomes, protection is limited to two alternating or consecutive DNA residues. The undigested portions correspond to 3′-labeled TNA of varying lengths. These results show that TNA can protect internal DNA residues from enzymatic degradation so long as the DNA residues follow an alternating pattern or short runs of 2 or less nucleotides.

Last, we examined the ability for TNA to protect RNA from enzyme-mediated degradation. To address this possibility, increasing amounts of complementary and non-complementary TNA were incubated with a target RNA strand in the presence of 0.5 mg/mL human liver microsomes. Under these conditions, denaturing PAGE analysis reveals that only the complementary TNA strand will protect the RNA strand from nuclease digestion, while the non-complementary TNA strand leads to RNA degradation (Fig. 5). RNA protection occurs when the stoichiometry of the RNA and TNA strands is ~1:1. This result is consistent with the interpretation that Watson–Crick base pairing shields the RNA strand from nuclease digestion by forming a chimeric duplex. The alternative explanation, which is that TNA protects RNA by binding to nucleases present in the biological mixture is unlikely, as the non-complementary TNA strand has no observable effect on RNA stability.

The efficacy of RNA-based therapeutics is dependent on high nuclease stability, as modified oligonucleotides must survive the harsh in vivo environment. In this regard, there is an ever-present search for new and better RNA analogues that function with favorable pharmcokinetic and pharmacodynamic properties. While a significant effort has been made to explore the ribose sugar, recent efforts in xeno-nucleic acids have opened the door to RNA analogues with backbone structures that are distinct from those found in nature. This effort has the potential to augment existing RNA therapeutics with properties that are not easily found in DNA and RNA (or close structural analogues thereof).

In conclusion, we have demonstrated that TNA exhibits strong nuclease stability under biologically relevant conditions. In particular, we show that TNA oligonucleotides are stable against 50% human serum and 0.5 mg/mL human liver microsomes and resistant to snake venom phosphodiesterase. Additionally, we also show that TNA will protect limited numbers of internal DNA residues from nuclease digestion and shield complementary RNA from RNA cleaving enzymes. Together, these observations warrant further analysis of TNA as a biologically stable analogue for RNA-based therapeutics.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.03.118.

References and notes

1. Burnett, J. C.; Rossi, J. J. *Chem. Biol.* **2012**, *19*, 60.
2. Deleavey, G. F.; Damha, M. J. *Chem. Biol.* **2012**, *19*, 937.
3. Bennet, C. F.; Swayze, E. E. *Ann. Rev. Pharmacol. Taxicol.* **2010**, *50*, 259.
4. Manoharan, M. *Biochim. Biophys. Acta* **1999**, *1489*, 117.
5. Keefe, A. D.; Pai, S.; Ellington, A. D. *Nat. Rev. Drug Discovery* **2010**, *9*, 537.
6. Freier, S. M.; Altmann, K.-H. *Nucleic Acids Res.* **1997**, *25*, 4429.
7. Ng, E. W. M.; Shima, D. T.; Calias, P.; Cunningham, E. T.; Guyer, D. R.; Adamis, A. P. *Nat. Rev. Drug Discovery* **2006**, *5*, 123.

8. Mi, J.; Liu, Y.; Rabbani, Z. N.; Yang, Z.; Urban, J. H.; Sullenger, B. A.; Clary, B. M. *Nat. Chem. Biol.* **2010**, *6*, 22.
9. Watts, J. K.; Corey, D. R. *J. Pathol.* **2012**, *226*, 365.
10. Yu, H.; Zhang, S.; Chaput, J. C. *Nat. Chem.* **2012**, *4*, 183.
11. Pinheiro, V. B.; Taylor, A. I.; Cozens, C.; Abramov, M.; Renders, M.; Zhang, S.; Chaput, J. C.; Wengel, J.; Peak-Chew, S. Y.; McLaughlin, S. H.; Herdewijn, P.; Holliger, P. *Science* **2012**, *336*, 341.
12. Joyce, G. F. *Science* **2012**, *336*, 307.
13. Schoning, K. U.; Scholz, P.; Guntha, S.; Wu, X.; Krishnamurthy, R.; Eschenmoser, A. *Science* **2000**, *290*, 1347.
14. Ebert, M.-O.; Mang, C.; Krishnamurthy, R.; Eschenmoser, A.; Jaun, B. *J. Am. Chem. Soc.* **2008**, *130*, 15105.
15. Orgel, L. E. *Science* **2000**, *290*, 1306.
16. Chaput, J. C.; Yu, H.; Zhang, S. *Chem. Biol.* **2012**, *19*, 1360.
17. Dunn, M. R.; Otto, C.; Fenton, K. E.; Chaput, J. C. *ACS Chem. Biol.* **2016**. http://dx.doi.org/10.1021/acschembio.5b00949.
18. Yu, H.; Zhang, S.; Dunn, M.; Chaput, J. C. *J. Am. Chem. Soc.* **2013**, *135*, 3583.
19. Barrett, S. E.; Abrams, M. T.; Burke, R.; Carr, B. A.; Crocker, L. S.; Garbaccio, R. M.; Howell, B. J.; Kemp, E. A.; Kowtoniuk, R. A.; Latham, A. H.; Leander, K. R.; Leone, A. M.; Patel, M.; Pechenov, S.; Pudvah, N. T.; Riley, S.; Sepp-Lorenzino, L.; Walsh, E. S.; Williams, J. M.; Colletti, S. L. *Int. J. Pharm.* **2014**, *466*, 58.
20. Noronha, A. M.; Wilds, C. J.; Lok, C. N.; Viazovkina, K.; Arion, D.; Parniak, M. A.; Damha, M. J. *Biochemistry* **2000**, *39*, 7050.
21. Cummins, L. L.; Owens, S. R.; Risen, L. M.; Lesnik, E. A.; Freier, S. M.; McGee, D.; Guinosso, C. J.; Cook, P. D. *Nucleic Acids Res.* **1995**, *2019*, 23.