Published in final edited form as:

Chembiochem. 2007 August 13; 8(12): 1359–1362.

Synthesis of a Universal 5-Nitroindole Ribonucleotide and Incorporation into RNA by a Viral RNA-Dependent RNA Polymerase

Daniel A. Harki^[a], Jason D. Graci^[b], Jocelyn P. Edathil^[a], Christian Castro^[b], Craig E. Cameron^[b], and Blake R. Peterson^[a]

[a] Dr. D. A. Harki, J. P. Edathil, Prof. B. R. Peterson, Department of Chemistry, The Pennsylvania State University, 104 Chemistry Building, University Park, PA 16802 (USA), Fax: (+1) 814-863-5319, E-mail: brpeters@chem.psu.edu

[b] J. D. Graci, Dr. C. Castro, Prof. C. E. Cameron, Department of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, PA 16802 (USA)

Keywords

antiviral agents; mutagenesis; nucleobases; poliovirus; polymerases; universal base

Small molecules that mimic natural nucleosides and nucleotides comprise a major class of antiviral agents. A new approach to the design of these compounds focuses on the generation of lethal mutagens:[1,2] compounds that further accelerate the high rate of viral mutagenesis [3,4] to confer antiviral effects. By incorporating artificial nucleobases with degenerate base-pairing abilities into viral genomes, lethal mutagens increase viral genomic mutagenesis to intolerable levels during replication, a process termed “error catastrophe”, which results in the loss of viral viability.[5,6] The antiviral drug ribavirin (1) is one such lethal mutagen effective against the RNA viruses poliovirus (PV) [7] and hepatitis C virus.[8] Ribavirin is converted intracellularly to the 5'-triphosphate (RTP), which is a substrate for viral RNA-dependent RNA polymerases (RdRP). By mimicking the natural purines, RTP is misincorporated opposite pyrimidines in the enzyme-bound viral RNA template. The incorporated nucleobase of ribavirin promotes genomic mutagenesis by templating C and U during subsequent rounds of viral replication; this facilitates error catastrophe and loss of viral viability.[7,9–11]

Correspondence to: Blake R. Peterson.

Supporting information for this article is available on the WWW under <http://www.chembiochem.org> or from the author.

As part of our efforts to identify more efficacious antiviral lethal mutagens, we report the synthesis, X-ray structure, and antiviral evaluation of the 5-nitroindole-containing ribonucleoside **3**, and incorporation of the related ribonucleotide **5** into RNA by a viral RdRP. These compounds represent RNA analogues of the previously reported “universal” deoxyribonucleoside **2**, a compound shown to base pair with all four natural DNA pseudobases. [12,13] By eliminating strong hydrogen-bond donors/acceptors, and possessing a large aromatic π system, 5-nitroindole **2** stabilizes DNA duplexes by aromatic π -stacking interactions with adjacent DNA bases.[14,15] The utility of **2** has been demonstrated in applications that range from incorporation into DNA hairpins,[16,17] primers for PCR and DNA sequencing, [13,18,19] detection of single nucleotide polymorphisms,[20,21] and (pseudobase) incorporation into peptide nucleic acids.[22]

We hypothesized that ribonucleoside analogues with universal base-pairing properties might possess enhanced antiviral activity relative to ribavirin (a purine mimic) by accelerating lethal viral mutagenesis. We demonstrate here that 5-nitroindole ribonucleotide **5** is universally incorporated opposite each native RNA base by a viral (poliovirus) RdRP (3D^{pol}). Although triphosphate **5** becomes incorporated into RNA by poliovirus 3D^{pol} more slowly than ribavirin triphosphate (RTP), **5** represents a much more potent inhibitor of this viral enzyme, and nucleoside **3** exhibits antiviral activity in cell culture.

5-Nitroindole ribonucleoside **3** and phosphorylated analogues **5–7** were synthesized as shown in Scheme 1. Commercially available **9** was chlorinated with TiCl₄, [23] treated with the sodium salt of 5-nitroindole, and globally deprotected with NH₃ in MeOH to yield the target nucleoside **3**. Triphosphate **5** was prepared by using the well-precedented phosphorylation conditions shown.[24] Monophosphate **7** was synthesized by modification of the conditions used to prepare **5**, [25] except the initially formed phosphodichloridate (step i) was immediately hydrolyzed with aqueous triethylammonium bicarbonate (TEAB). Diphosphate **6** was elaborated from monophosphate **7** by CDI activation of the α -phosphate to yield a phosphoimidazolidate intermediate,[26] which was subsequently displaced with phosphate to install the β -phosphate of **6**. The structure and stereochemistry of **3** were confirmed by X-ray crystallography (Figure 1). [27] Other methods reported to yield **3** were attempted but did not yield the desired compound [28] or did not provide the pure β -anomer.[29]

To investigate whether the 5-nitroindole pseudobase was recognized and incorporated universally by a viral RdRP, we subjected triphosphate **5** to a primer-extension assay[30] with recombinant poliovirus RdRP (3D^{pol}). [31] This assay provides kinetic and thermodynamic measurements of nucleotide incorporation into a symmetrical primer/template (designated s/s) by viral RdRPs. As shown in Figure 2, the incorporation of **5** into RNA by 3D^{pol} was evaluated across the four naturally occurring nucleotides (A, C, G, U) in the RNA template. Detailed kinetic analysis of incorporation of **5** into s/s-U afforded the data listed in Table 1. These experiments revealed that triphosphate **5** was incorporated across all four natural templating nucleotides and demonstrate its ability to function as a universal base. Kinetic measurements with the s/s-U template revealed **5** was incorporated tenfold more slowly than ribavirin triphosphate, yet tenfold more rapidly than a previously reported structurally-related 3-nitropyrrole ribonucleotide.[32] Although the kinetic data suggest that natural nucleoside triphosphates (NTPs) will be incorporated much more efficiently than **5** into viral RNA by PV RdRP, the sensitivity of viral genomes to even subtle increases in mutation frequency[10] renders them vulnerable to even low levels of incorporation of lethal mutagens. For example, ribavirin triphosphate incorporates into viral RNA with a frequency similar to natural nucleotide misincorporation.[7]

Previous studies that measured incorporation of 5-nitroindole 2'-deoxyribonucleotide (**4**) opposite native DNA bases by DNA polymerases (pol α and *E. coli* KF) revealed that **4**

functions as a universal DNA nucleotide. However, further extension past the templated 5-nitroindole pseudobase was not observed for either polymerase; this suggests that **4** also functions as a chain terminator.[33,34] To examine whether **5** functions as a chain terminator following viral incorporation by RdRP, an additional primer-extension assay with PV RdRP and the s/s-U RNA template was performed. In this experiment, enzyme-bound Mg^{2+} was replaced with Mn^{2+} to decrease RdRP fidelity and overcome the slow rate of incorporation of **5** relative to the rate of enzyme dissociation.[35] Extension of the RNA primer/template by up to three nucleotides (Figure 3) was observed under these conditions; this is consistent with a lack of chain termination activity.

PV RdRP incorporated **5** into RNA very slowly relative to the “correct” nucleotides[36] and with a low apparent K_d (Table 1). If this K_d represents binding to the enzyme active site, then **5** should inhibit elongation catalyzed by the RdRP. To probe for polymerase inhibition by **5**, primer extension by PV RdRP was assayed by using stopped-flow kinetics with the s/s-U RNA template, which contained 2-aminopurine (2AP) as a fluorescent probe. Inhibitors of incorporation of the natural substrate ATP were analyzed (Figure 4). Inhibition constants (K_i) were determined by simulation against a competitive inhibition model (Figure S1). As shown in Table 1, ribonucleotide **5** inhibited PV RdRP ($3D^{pol}$) approximately five times more potently than RTP. To investigate whether related 5'-phosphates **6** and **7** could similarly inhibit $3D^{pol}$, we subjected both compounds to the same assay conditions. Interestingly, diphosphate **6** was found to strongly inhibit PV RdRP ($K_i = 50 \mu M$) whereas monophosphate **7** and nucleoside **3** lacked any detectable inhibitory activity. These experiments suggest that the diphosphate group is a critical determinant of inhibition, and the terminal γ -phosphate substituent plays a relatively minor role in binding and inhibition of $3D^{pol}$ by phosphorylated analogues of **3**.

Human HeLa cells infected with poliovirus were treated with ribonucleoside **3** and ribavirin (**1**) to examine the biological activities of these compounds in cell culture. Antiviral activity was compared with effects on proliferation of the host cell line (Figure 5). Importantly, both **3** and **1** substantially reduced the titer of poliovirus. Moreover, coadministration of the cytochrome P-450 inhibitor sulconazole[37] (**8**) with **3** magnified the antiviral activity of **3**, presumably by affecting metabolism of the nitroindole base. However, **8** did not affect the activity of **1**. The combination of **3** (1 mM) and **8** (10 μM) reduced viral titer by over two orders of magnitude; this surpasses the antiviral activity of **1** by approximately fivefold at this concentration. Only a slight effect on the proliferation of the HeLa host cells was observed at the highest dose evaluated.

We conclude that PV RdRP can incorporate a ribonucleotide that bears the 5-nitroindole pseudobase into RNA opposite each templating base. Although the rate of incorporation of triphosphate **5** into RNA by PV RdRP was slower than RTP and natural nucleoside triphosphates, both **5** and diphosphate **6** were much more potent inhibitors of this enzyme. Ribonucleoside **3** reduced the titer of poliovirus in cell culture, and this compound represents a promising lead for the development of novel antiviral lethal mutagens and related inhibitors of viral RdRPs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Dr. H. Yennawar (Penn State University) for X-ray crystallography. We thank the NIH (AI054776 to B.R.P. and C.E.C.), the American Heart Association (0340028N to C.E.C. and predoctoral fellowships to D.A.H. and J.P.E.), and the NSF (CHE-0131112 funding for the X-ray facility) for financial support.

References

1. Loeb LA, Essigmann JM, Kazazi F, Zhang J, Rose KD, Mullins JI. *Proc Natl Acad Sci USA* 1999;96:1492–1497. [PubMed: 9990051]
2. Loeb LA, Mullins JI. *AIDS Res Hum Retroviruses* 2000;16:1–3. [PubMed: 10628810]
3. Drake JW, Holland JJ. *Proc Natl Acad Sci USA* 1999;96:13910–13913. [PubMed: 10570172]
4. Domingo E, Escarmis C, Sevilla N, Moya A, Elena SF, Quer J, Novella IS, Holland JJ. *FASEB J* 1996;10:859–864. [PubMed: 8666162]
5. Anderson JP, Daifuku R, Loeb LA. *Annu Rev Microbiol* 2004;58:183–205. [PubMed: 15487935]
6. Freistadt MS, Meades GD, Cameron CE. *Drug Resist Updates* 2004;7:19–24.
7. Crotty S, Maag D, Arnold JJ, Zhong W, Lau JYN, Hong Z, Andino R, Cameron CE. *Nat Med* 2000;6:1375–1379. [PubMed: 11100123]
8. Maag D, Castro C, Hong Z, Cameron CE. *J Biol Chem* 2001;276:46094–46098. [PubMed: 11602568]
9. Cameron CE, Castro C. *Curr Opin Infect Dis* 2001;14:757–764. [PubMed: 11964896]
10. Crotty S, Cameron CE, Andino R. *Proc Natl Acad Sci USA* 2001;98:6895–6900. [PubMed: 11371613]
11. Vignuzzi M, Stone JK, Andino R. *Virus Res* 2005;107:173–181. [PubMed: 15649563]
12. Loakes D, Brown DM. *Nucleic Acids Res* 1994;22:4039–4043. [PubMed: 7937128]
13. Loakes D, Hill F, Linde S, Brown DM. *Nucleosides Nucleotides* 1995;14:1001–1003.
14. Wheaton CA, Dobrowolski SL, Millen AL, Wetmore SD. *Chem Phys Lett* 2006;428:157–166.
15. Gallego J, Loakes D. *Nucleic Acids Res* 2007;35:2904–2912. [PubMed: 17438041]
16. Loakes D, Hill F, Brown DM, Salisbury SA. *J Mol Biol* 1997;270:426–435. [PubMed: 9237908]
17. Vallone PM, Benight AS. *Nucleic Acids Res* 1999;27:3589–3596. [PubMed: 10446251]
18. Loakes D, Brown DM, Linde S, Hill F. *Nucleic Acids Res* 1995;23:2361–2366. [PubMed: 7630712]
19. Loakes D, Hill F, Brown DM, Ball S, Reeve MA, Robinson PS. *Nucleosides Nucleotides* 1999;18:2685–2695.
20. Burgner D, D'Amato M, Kwiatkowski DP, Loakes D. *Nucleosides Nucleotides Nucleic Acids* 2004;23:755–765. [PubMed: 15281364]
21. Frutos AG, Pal S, Quesada M, Lahiri J. *J Am Chem Soc* 2002;124:2396–2397. [PubMed: 11890762]
22. Challa H, Styers ML, Woski SA. *Org Lett* 1999;1:1639–1641. [PubMed: 10836026]
23. Schramm, VL.; Furneaux, RH.; Tyler, PC.; Clinch, K. US Patent. 6,379,911 B2. 2002.
24. Burgess K, Cook D. *Chem Rev* 2000;100:2047–2059. [PubMed: 11749283]
25. Major DT, Laxer A, Fischer B. *J Org Chem* 2002;67:790–802. [PubMed: 11856021]
26. Hecht SM, Kozarich JW. *Biochim Biophys Acta* 1973;331:307–309. [PubMed: 4777661]
27. CCDC-642 211 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif
28. Mukhanov VI, Miniker TD, Preobrazhenskaya MN. *Zh Org Khim* 1977;13:214–218.
29. Gaubert G, Wengel J. *Tetrahedron Lett* 2004;45:5629–5632.
30. Arnold JJ, Cameron CE. *J Biol Chem* 2000;275:5329–5336. [PubMed: 10681506]
31. Gohara DW, Ha CS, Ghosh SKB, Arnold JJ, Wisniewski TJ, Cameron CE. *Protein Expression Purif* 1999;17:128–138.
32. Harki DA, Graci JD, Korneeva VS, Ghosh SKB, Hong Z, Cameron CE, Peterson BR. *Biochemistry* 2002;41:9026–9033. [PubMed: 12119016]
33. Chiamonte M, Moore CL, Kincaid K, Kuchta RD. *Biochemistry* 2003;42:10472–10481. [PubMed: 12950174]
34. Smith CL, Simmonds AC, Felix IR, Hamilton AL, Kumar S, Nampalli S, Loakes D, Hill F, Brown DM. *Nucleosides Nucleotides* 1998;17:541–554.
35. Arnold JJ, Gohara DW, Cameron CE. *Biochemistry* 2004;43:5138–5148. [PubMed: 15122879]
36. Arnold JJ, Cameron CE. *Biochemistry* 2004;43:5126–5137. [PubMed: 15122878]

37. Zhang WJ, Ramamoorthy Y, Kilicarslan T, Nolte H, Tyndale RF, Sellers EM. Drug Metab Dispos 2002;30:314–31. [PubMed: 11854151]

Figure 1.
X-ray structure of **3** (ORTEP representation, 50 % probability).[27]

Figure 2.

Incorporation of ribonucleotides into double-stranded RNA by poliovirus RdRP (3D^{pol}). Complexes of s/s-N RNA template (N represents the first templating nucleobase of the RNA primer) and 3D^{pol} were incubated for 90 s, the complementary NTP (0.1 mM) was added, and the reaction was allowed to proceed for 15 min at 30 °C; Nuc.: nucleotide.

Figure 3.

Chain-termination assay. The s/s-U template was used for primer extension by the PV RdRP (3D^{pol}) in the presence of Mn²⁺. Ribonucleotide **5** (1 mM) and UTP (10 μM) were provided as substrates for the extension reaction.

Figure 4.

Stopped-flow kinetic analyses of inhibition of incorporation of ATP catalyzed by PV RdRP. Best-fit lines (nonlinear regression to a single exponential) of raw fluorescence data (see the Supporting Information) are shown. $[3D^{Pol}] = 0.5 \mu M$; $[s/s-U-2AP] = 0.25 \mu M$ (duplex); $[ATP]$, $[RTP]$, $[5]$, and $[6] = 100 \mu M$. Data for **6** were extrapolated to 0.25 s (last data point collected at 0.20 s).

Figure 5.

Antiviral and antiproliferative activity of **3** compared with ribavirin (**1**) in the presence and absence of sulconazole (**8**; 10 μ M). A) Effects on the titer of poliovirus in infected HeLa cells (7 h treatment). B) Cytotoxicity of compounds to HeLa cells measured by using Trypan blue exclusion assay (7 h treatment).

Scheme 1.

a) TiCl_4 , CH_2Cl_2 , 23 °C, 2 h; b) 5-nitroindole, NaH, MeCN, 2 °C–23 °C, 22 h; c) NH_3 , MeOH, 50 °C (sealed tube), 12 h; d) i. POCl_3 , $(\text{CH}_3\text{O})_3\text{P}=\text{O}$, Proton Sponge, 2 °C, 2 h; ii. Bu_3N , tributylammonium pyrophosphate, DMF, 2 °C, 2 min; TEAB hydrolysis; e) POCl_3 , $(\text{CH}_3\text{O})_3\text{P}=\text{O}$, Proton Sponge, 2 °C, 2 h; TEAB hydrolysis; f) i. Bu_3N , CDI, DMF, 23 °C, 3 h; MeOH; ii. anhydrous H_3PO_4 , Bu_3N , DMF, 23 °C, 12 h. Proton Sponge: 1,8-bis(dimethylamino)naphthalene; TEAB: triethylammonium bicarbonate; CDI: carbonyl diimidazole.

Table 1Kinetics of incorporation into dsRNA and inhibition of PV 3D^{pol} by nucleoside 5'-phosphates.^[a]

NS'-P ^[b]	$K_{d,app}$ [μ M] ^[c]	k_{pol} [s ⁻¹] ^[c]	K_i [μ M] ^[d]
5	9.9 $\pm$ 1.5	0.00136 $\pm$ 0.00006	30
6	n.d. ^[e]	n.d. ^[e]	50
RTP ^[b]	496 $\pm$ 21 ^[f]	0.014 $\pm$ 0.001 ^[f]	150

^[a] See the Supporting Information for experimental details.^[b] NS'-P: nucleoside 5'-phosphate; RTP: ribavirin 5'-triphosphate.^[c] Kinetic analysis of nucleotide incorporation into s/s-U template (Figure 2) by recombinant PV 3D^{pol} measured by using the primer-extension assay. [30,31]^[d] Stopped-flow, fluorescence-based kinetic analysis of nucleotide incorporation inhibition by NTPs (Figure 4).^[e] Not determined.^[f] Previously reported values.[7]