Rapid incorporation kinetics and improved fidelity of a novel class of 3’-OH unblocked reversible terminators

Andrew F. Gardner1, Jinchun Wang2, Weidong Wu2, Jennifer Karouby1, Hong Li2, Brian P. Stupi2, William E. Jack1, Megan N. Hersh2 and Michael L. Metzker2,3,4,*

1New England Biolabs, Ipswich, MA 01938, 2LaserGen, Inc., Houston, TX 77054, 3Human Genome Sequencing Center and 4Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030, USA

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ABSTRACT

Recent developments of unique nucleotide probes have expanded our understanding of DNA polymerase function, providing many benefits to techniques involving next-generation sequencing (NGS) technologies. The cyclic reversible termination (CRT) method depends on efficient base-selective incorporation of reversible terminators by DNA polymerases. Most terminators are designed with 3’-O-blocking groups but are incorporated with low efficiency and fidelity. We have developed a novel class of 3’-OH unblocked nucleotides, called Lightning Terminators™, which have a terminating 2-nitrobenzyl moiety attached to hydroxymethylated nucleobases. A key structural feature of this photocleavable group displays a ‘molecular tuning’ effect with respect to single-base termination and improved nucleotide fidelity. Using Therminator™ DNA polymerase, we demonstrate that these 3’-OH unblocked terminators exhibit superior enzymatic performance compared to two other reversible terminators, 3’-O-amino-TTP and 3’-O-azidomethyl-TTP. Lightning Terminators™ show maximum incorporation rates ($k_{pol}$) that range from 35 to 45 nt/s, comparable to the fastest NGS chemistries, yet with catalytic efficiencies ($k_{pol}/K_D$) comparable to natural nucleotides. Pre-steady-state kinetic studies of thymidine analogs revealed that the major determinant for improved nucleotide selectivity is a significant reduction in $k_{pol}$ by >1000-fold over TTP misincorporation. These studies highlight the importance of structure–function relationships of modified nucleotides in dictating polymerase performance.

INTRODUCTION

DNA polymerases belong to at least seven families [A, B, C, D, X, Y and reverse transcriptase (RT)] that display a wide range of cellular roles, replicative fidelities and propensities toward incorporating modified nucleotide substrates or natural nucleotides across damaged templating bases (1–5). For example, high-fidelity DNA polymerases have a remarkable ability to copy their genomes with high accuracy. For DNA polymerases in general, a major factor contributing to the degree of replicative fidelity is nucleotide selectivity; that is, the ability for a DNA polymerase to correctly choose the right nucleotide substrate over the wrong one. The capacity by which a DNA polymerase performs this selection process occurs by recognizing at least two differences in the nucleotide structure: (i) sugar selectivity (i.e. an OH group versus an H atom at the 2’-position of ribose) and (ii) nucleobase selectivity (i.e. geometric shape of the Watson–Crick versus mismatched base-pairs).

Despite low sequence conservation, there is a striking similarity in the overall structural architecture among many polymerase families, reminiscent of a half-opened right hand containing the fingers, palm and thumb domains (6,7). Protein sequence alignment for polymerase members belonging to Families A, B and RT have revealed two highly conserved regions, motif A and motif C, which participate in nucleotide binding and the phosphoryl transfer reaction within the fingers and palm domains of the active site (8). Key amino acid residue(s) within motif A of DNA polymerases belonging to these families have been shown to act as a ‘steric gate’ to discriminate against the incorporation of ribonucleotides by a structural clash with the 2’-OH group (9–15). Interestingly, mutational analysis of amino acid residues involving or immediately surrounding the steric gate region of motif A have also revealed lower DNA synthesis fidelity (16–19) and an
increased capability to extend synthesis beyond mismatched incorporated bases (20). Nucleobase discrimination is thought to involve a tight fit of the overall geometric shape of the correct Watson–Crick base-pair within the closed active site to promote the fast catalytic activity of DNA polymerase (4,21–24). Thus, the relaxation of sugar selectivity may be coupled with lower fidelity by having a looser fit that accommodates larger geometric shapes of mismatched base-pairs in the closed active site.

Easing of this otherwise structural stringency with appropriate steric gate variants is not limited to the 2'-position of nucleotides. For example, DNA polymerases with several steric gate variants have also been shown to improve the incorporation of 2',3'-dideoxynucleoside-5'-triphosphates (2',3'-ddNTPs) (12,25). These 2',3'-ddNTPs have been widely used in Sanger sequencing (26) and served as the initial basis to develop another class of nucleotide terminators, 3'-O-modified reversible terminators (27,28). These 3'-O-blocked nucleotides are currently being used in several next-generation sequencing (NGS) technology approaches by employing the cyclic reversible termination (CRT) method (29,30), which involves single-base incorporation, fluorescent imaging to identify the just incorporated base, and removal of the terminating and fluorescent dye moieties to restore the 3'-OH group. A number of 3'-O-modified reversible terminator chemistries have been developed with labile blocking groups such as 3'-O-amino (31,32), 3'-O-azidomethyl (33,34), 3'-O-allyl (27,35,36) and 3'-O-nitrobenzyl (36,37). A major challenge with this approach has been to identify appropriate DNA polymerases that can incorporate the 3'-O-modified nucleotide with the desired properties of fast nucleotide-incorporation kinetics and high nucleotide selectivity (30).

Our group was the first to demonstrate that a small 2-nitrobenzyl group attached to the N6-position of dATP created an effective reversible terminator with wild-type Bst DNA polymerase (37). Upon photochemical cleavage with 365 nm ultraviolet (UV) light, this 2-nitrobenzyl-modified dATP analog is transformed back into its corresponding natural nucleotide form. While this 3'-OH unblocked nucleotide was a promising reversible terminator, translation of this approach to an N7-modified thymidine analog was predicted and confirmed to adversely affect nucleotide-incorporation fidelity. This prompted development of novel 2-nitrobenzyl-modified thymidine analogs based on 5-hydroxymethyl-2'-deoxyuridine-5'-triphosphate (HOMedUTP), a naturally found hypermodified nucleotide (38).

We have demonstrated a ‘molecular tuning’ effect by increasing the size of the alkyl group attached to the α-methylene carbon of the 2-nitrobenzyl group to confer unique properties such as high nucleotide selectivity and single-base termination with two commercially-available DNA polymerases, Therminator and Vent(exo-) (38). The 3'-OH unblocked analog 5'-(S)-1-(2-nitrophenyl)-2,2-dimethyl-propylxymethyl-dUTP (dU.V) was identified as an efficient reversible terminator by repeated cycles of stepwise incorporation and photochemical cleavage through a homopolymer repeat of ten complementary templating bases (38) using the CRT method (29,30). A complete set of 3'-OH unblocked reversible terminators based on 7-deaza-7-hydroxymethyl-2'-deoxyadenosine-5'-triphosphate (C'-HOMedATP), 5-hydroxymethyl-2'-deoxy cytidine-5'-triphosphate (HOMedCTP) and 7-deaza-7'-hydroxymethyl-2'-deoxy-guanosine-5'-triphosphate (C'-HOMe-dGTP) has now been developed, called Lightning TerminatorsTM. We have demonstrated that the stereo-specific S configuration of an α-tert-butyl group and the ring modification of a 5-OMe group are important determinants in providing fast photochemical cleavage kinetics (39). To further explore the unique properties of these 3'-OH unblocked reversible terminators, we have now characterized the pre-steady-state kinetic basis of 5'-hydroxymethyl-2'-deoxycytosine (HOMedU) analog dU.V and its 5-OMe substituted analog 5'-(S)-1-(5-methoxy-2-nitrophenyl)-2,2-dimethyl-propylxymethyl-dUTP (dU.VI, Figure 1A), having the properties of fast nucleotide-incorporation kinetics, high nucleotide selectivity and single-base termination. We present evidence that these 3'-OH unblocked reversible terminators exhibit superior enzymatic performance properties when compared with two 3'-O-blocked reversible terminators, namely 3'-O-amino-TTP and 3'-O-azidomethyl-TTP (Figure 1B) using Terminator DNA polymerase. Remarkably, the other nucleotide members of the Lightning TerminatorsTM set (i.e. dA.VI, dC.VI and dG.VI, Figure 1C) show fast nucleotide-incorporation kinetics similar to its thymidine counterpart.

MATERIALS AND METHODS
DNA polymerases and nucleic acids
9N'-7(exo-) DNA polymerase is a Family B DNA polymerase, cloned from the Thermococcus species 9N'-7, and contains the D141A and E143A variants causing 3'→5' exonuclease deficiency (40). Therminator DNA polymerase is 9N'-7(exo-) that also contains the A485L variant (41). Therminator III DNA polymerase is 9N'-7(exo-) DNA polymerase that also contains the L408S, Y409A and P410V variants. Therminator, Therminator III and Klenow(exo-) DNA polymerases, along with TTP, were obtained from New England Biolabs, Inc. (Ipswich, MA, USA). HOMedUTP (38) was purchased from TriLink BioTechnologies, Inc. (San Diego, CA, USA). 3'-O-amino-TTP (31) was purchased from Firebird Biomolecular Sciences, LLC (Gainesville, FL, USA). Other nucleotide analogs were synthesized as previously described: 3'-O-azidomethyl-TTP (33,42), 5'-(S)-1-(2-nitrophenyl)-2,2-dimethyl-propylxymethyl-dUTP (dU.V) (38) and 7'-(S)-1-(5-methoxy-2-nitrophenyl)-2,2-dimethyl-propylxymethyl-7-deaza-dATP (dA.VI), 5'-(S)-1-(5-methoxy-2-nitrophenyl)-2,2-dimethyl-propylxymethyl-dCTP (dC.VI), 7'-(S)-1-(5-methoxy-2-nitrophenyl)-2,2-dimethyl-propylxymethyl-7-deaza-dGTP (dG.VI) and 5'-(S)-1-(5-methoxy-2-nitrophenyl)-2,2-dimethyl-propylxymethyl-dUTP (dU.VI) (39). Oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA, USA).
All thymidine nucleotides except for 3'-O-amino-TTP were purified by reverse-phase high performance liquid chromatography (RP–HPLC) as previously described (38,39), followed by enzymatic ‘Mop-Up’ to remove contaminating TTP or HOMedUTP (43). A successful mop-up was followed by enzymatic ‘Mop-Up’ to remove contaminating TTP. Following a single round of RP–HPLC, bead-based mop-up assays were performed using Therminator DNA polymerase. Eliminating TTP contamination from the 3'-O-amino-TTP stock proved difficult as these nucleotides comigrate by RP–HPLC, consistent with that noted by Benner (44). As 3'-O-amino-TTP is incorporated by many commercially-available DNA polymerases (32), we devised a solution-based, kinetic mop-up assay using Therminator DNA polymerase. Eliminating TTP contamination from the 3'-O-amino-TTP stock proved difficult as these nucleotides comigrate by RP–HPLC, consistent with that noted by Benner (44). As 3'-O-amino-TTP is incorporated by many commercially-available DNA polymerases (32), we devised a solution-based, kinetic mop-up assay using Therminator DNA polymerase by incubating the reaction at 65°C for 1 min. This is based on the premise that the incorporation rate of TTP is faster than that of 3'-O-amino-TTP and short incubation times would effectively remove much of the contaminating natural nucleotide. The 3'-O-azidomethyl-TTP analog was purified three times by RP–HPLC followed by the kinetic mop-up assay to remove contaminating TTP. Following a single round of RP–HPLC, bead-based mop-up assays were performed using Klenow (exo⁻) DNA polymerase as the mop-up enzyme for dU.V and dU.VI (43). In all cases, mopped-up solutions were separated from the magnetic beads (bead-based method), passed through an Amicon Ultra-0.5 centrifugal 10 000 molecular weight cut-off (MWCO) filter (Millipore, Billerica, MA, USA) to remove mop-up polymerase and primer/template duplex, and placed on ice in the dark. The other Lightning Terminators™ (dA.VI, dC.VI and dG.VI) were also purified by RP–HPLC, but were not subjected to the mop-up assay as these purified samples were determined to be free of natural nucleotide contamination by incorporation assays, as described above.

Polymerase end-point assays using correct and mismatched templating bases
Polymerase end-point (PEP) assays for incorporation (correct) and nucleotide selectivity (mismatched) experiments were performed as previously described (37,38). Incorporation reactions were analyzed on a 10% polyacrylamide gel using an Applied Biosystems (AB), now Life Technologies (Foster City, CA, USA) model 377 DNA sequencer and informative fluorescence peaks were quantified manually.

Burst kinetics assays
FAM-duplex DNA-A was formed by mixing equimolar amounts of the 6-carboxyfluorescein-labeled primer, 5'-A GTGAAAATTCGAGCTCGGTACCCGGGATCCCTCTA GAGTCGACCTGAGGG, with the template-A, 5'-TT GCTCGTTTGGCTGGAGCTCGAGTCGACTCTA GAGGATCCCGGGGTACCCGAGCTCGAATTTCGACT, (interrogation base underlined) in 1× Thermopol Buffer [20 mM Tris–HCl, 10 mM (NH₄)₂SO₄, 10 mM KCl, 2 mM MgSO₄, 0.1% Triton X-100, pH 8.8] and then by heating to 95°C for 5 min, followed by annealing the duplex at

![Figure 1. Chemical structures of (A) 3'-OH unblocked reversible terminators: dU.V and dU.VI, (B) 3'-O-modified reversible terminators: 3'-O-amino-TTP and 3'-O-azidomethyl-TTP and (C) 3'-OH unblocked reversible terminators: dA.VI, dC.VI and dG.VI. Red chemical structures denote terminating functional groups that cleave when exposed to (A and C) UV light or (B) sodium nitrite or tris(2-carboxyethyl)phosphine, respectively.](image-url)
60°C for 10 min, and cooling to room temperature over a 15 min period. FAM-duplex DNA-C, -G and -T were also assayed by replacing template-A with template-C, 5'-AAGATATGAAAGTAGGGCCGCTGAGTGCGAC TCTAGAGGTCCCGGTACCCGAGCTCGAATTCACT, template-G, 5'-CCCTAATTACATCTCCTGGGCT GGAGTTCTAGTTAGAGTCCCGCCGAGCTCGAATTCACT, or template-T, 5'-AAGAGCCGAAAGGGTGTCGAGCTTGACACTCTAGAG CATCCCCGGTACCCGAGCTCGAATTCACT, respectively (interrogation base underlined).

Burst kinetic experiments were employed to determine the rate limiting step qualitatively. Rapid-quench reactions were carried out as described below by mixing 40 μl of 80 nM FAM-duplex DNA-A containing 20 nM Terminator DNA polymerase in 1× ThermoPol buffer with 40 μl of either 50 μM dU.V or dU.VI or 200 μM TTP or HOMedUTP in 1× ThermoPol buffer. FAM duplex DNA -C, -G, or -T reactions were similarly carried out with 50 μM dG.VI, dC.VI, or dA.VI, respectively. The reactions were allowed to proceed for pre-determined time points and then were quenched by addition of 200 μl of 0.5 M Na2EDTA. Time point increments that ranged from 3 ms to 10 s were performed using an RQF-3 rapid-quenched-flow instrument (Kintek Corp., Austin, TX, USA). Initial time points > 10 s were performed manually. All Terminator or Terminator III DNA polymerase reactions were analyzed at 60°C due to constraints of the RQF-3 instrument. Incorporation reactions were analyzed using an AB model 3730xl capillary analyzer. Fluorescence peaks were quantified and analyzed using Peak Scanner Software v1.0 (AB).

The burst amplitude (A), burst rate (kburst) and observed steady-state rate (kSS) were fitted using a non-linear least squares approach from the equation (45,46):

\[
\text{[product]} = A[1 - \exp(-k_{\text{burst}}t) +\frac{k_{\text{SS}}}{t}]
\]

The normalized rate constant (kSS) was then calculated by dividing kSS by A, having the units of nanomolar product formed per second per nanomolar active enzyme.

Pre-steady-state kinetic experiments

Single turnover nucleotide-incorporation reactions were performed by rapidly mixing 40 μl of 25 nM of Terminator DNA polymerase with 10 nM FAM-duplex DNA-A in 1× ThermoPol buffer together with 40 μl of nucleotides or nucleotide analogs at appropriate concentrations in 1× ThermoPol buffer. As described above, reactions proceeded for pre-determined time points, quenched with Na2EDTA and analyzed using an AB model 3730xl DNA capillary analyzer.

The first-order rate constant (kobs) for incorporation reactions for each nucleotide concentration was calculated by plotting the natural log of the remaining substrate versus time. Rate constants (kobs) were then plotted as a function of nucleotide or analog concentration and fitted to the hyperbolic equation:

\[
k_{\text{obs}} = \frac{k_{\text{pol}}[\text{nucleotide}]}{K_{\text{D}} + [\text{nucleotide}]}
\]

where kpol is the maximum rate of nucleotide addition and KD is the nucleotide ground state binding equilibrium constant (47).

Termination experiments

4,4-Difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-3-propionic acid (BODIPY-FL) duplex DNA-E was formed by mixing 40 nM of template-E, 5'-CCGACGTACGTAACTGGCCGCTGTTTTCAGCCGGCCGCGCGCGGGAGCTCGAATTCACT, or template-T, 5'-AAGACCGAAAGGGTGTCGAGCTTGACACTCTAGAGCATCCCCGGTACCCGAGCTCGAATTCACT, respectively (interrogation base underlined) with 5 nM of BODIPY-FL-labeled primer R931 (48), 5'-CGTAAAACGACGGCCAG-s-T (where ‘s’ is a phosphorothioate linkage) in 1× ThermoPol buffer and then by heating to 80°C for 30 s, followed by annealing the duplex at 57°C for 30 s, and then cooling to 4°C. The duplex was then diluted in half (its final concentration was 2.5 nM in a volume of 10 μl) by the addition of 94 nM Terminator DNA polymerase in 1× ThermoPol buffer. After heating at 65°C for 30 s, 1 μl of 100 μM dU.V or dU.VI was added. Incorporation reactions were rapidly mixed and incubated at 65°C for 0.5, 1, 2, 3, 4, 5, 10 and 20 min time increments, quenched with 10 μl of stop solution (98% deionized formamide; 10 nM Na2EDTA, pH 8.0; 25 mg/ml Blue Dextran, MW 2000000) and then placed on ice in the dark. Termination reactions were analyzed using an AB model 377 DNA sequencer, and informative fluorescence peaks were quantified manually.

RESULTS

Reversible terminators used in NGS technologies

The performance of the reversible terminator plays a critical role in cycle time and data quality for NGS systems (30). We recently described a novel set of 3'-OH unblocked terminators based on 2-nitrobenzyl-modified HOMedU nucleotides that were efficiently incorporated using either Vent(exo–) or Terminator DNA polymerases (38). For the current study, we chose Terminator DNA polymerase as it has been reported to be more tolerant to incorporating sugar-modified nucleotides belonging to the general classes of acyclic (41,49), ring-modified (50,51) and 3'-O-blocked (32) analogs. Thus, to characterize performance differences between 3'-O-blocked and 3'-OH unblocked terminators, we describe detailed enzymatic studies using Terminator DNA polymerase comparing the HOMedU analogs dU.V (38) and dU.VI (39) along with the thymidine analogs 3'-O-amino-TTP (31,32) and 3'-O-azidomethyl-TTP (33,34); see Figure 1A and B. Additionally, the other Lightning Terminators, dA.VI, dC.VI and dG.VI, (Figure 1C) were also tested to evaluate potential differences between nucleobase analogs.

A challenge of utilizing reversible terminators in NGS technologies is the presence of contaminating natural nucleotides (29). DNA polymerases generally discriminate against modified nucleotides in favor of their natural nucleotide counterparts when presented as a mixture. We refer to this property as the incorporation bias (37). This is important as interpretation of the results obtained from enzymatic experiments can be confounded when even...
Reduced incorporation bias with 3'-OH unblocked Lightning Terminators™

Initially, we performed the PEP assay (37) to measure the incorporation bias, expressed as the ratio of IC50 values (i.e. the nucleotide concentration at which the number of moles of the primer equals that of the incorporated product) for the modified nucleotide over the natural nucleotide using Therminator DNA polymerase. Compared with the IC50 value of HOMedUTP, dU.V and dU.VI each showed a slight incorporation bias of 2.9 and 4.3, respectively (Table 1). The incorporation bias for 3'-O-aminotTP was 9.0 and 10.1 compared with TTP and HOMedUTP, respectively. The bias for 3'-O-aminotTP was >2-fold higher compared to either 3'-OH unblocked terminator despite that the (S)-α-tert-butyl-2-nitrobenzyl group being substantially larger in size (Figure 1A and B). This comparison highlights the adverse effect of attaching even a small functional group such as NH2 to the 3'-O position of the nucleotide. This point is emphasized further with the modified TTP analog containing the larger 3'-O-azidomethyl group, which was not incorporated at all by Therminator DNA polymerase up to a final concentration of 10 μM (data not shown). These data are consistent with the inverse relationship of increasing size at the 3'-O-position and decreasing incorporation performance of Therminator DNA polymerase (i.e. –OH < –ONH2, >> –OCH2N3).

PEP assays were also performed with dA.VI, dC.VI and dG.VI and compared with the corresponding native nucleotides C2′-HOMedATP, HOMedCTP and C2′-HOMedGTP, respectively (Table 1), each revealing a lower incorporation bias (range 1.0–3.5) compared with that of dU.VI. These data highlight that low incorporation bias is an intrinsic property of Lightning Terminators™.

Lightning Terminators™ exhibit fast nucleotide-incorporation rates and higher binding affinities

We next performed pre-steady-state kinetic experiments to determine the constants kpol and KD for TTP, HOMedUTP, dU.V, dA.VI, dC.VI, dG.VI and dU.VI. Unfortunately, at concentrations >10 μM, TTP contamination could not be efficiently removed from the 3'-O-amino-TTP solution and given its incorporation bias of ~10-fold, it was not characterized further.

An incorporation event can be expressed by the following simplified kinetic reaction:

$$E + DNAn = E \cdot DNAn + dNTP \rightleftharpoons K_D$$

$$E \cdot DNAn + dNTP \overset{k_{pol}}{\rightleftharpoons} E \cdot DNA_{n+1} \cdot PP_i$$

where KD is the ground state nucleotide binding equilibrium constant and kpol is the maximum rate of incorporation, which encompasses both the conformation change from the open to closed state (k2) and the phosphoryl transfer chemistry step (k3) (4).

Pre-steady-state kinetic studies were performed with Therminator DNA polymerase in molar excess over the primer/template duplex. Molar excess was verified by repeating these experiments at a 2-fold higher Therminator DNA polymerase concentration, which did not alter either kpol or KD constants (data not shown). The kinetic constants, kpol and KD, for TTP and HOMedUTP were similar to those for dCTP (45) using the related archaeal DNA polymerases, Vent(exo−) and Vent(exo+) A488L variant, the latter of which contains the amino acid substitution analogous to that found in Therminator DNA polymerase (Table 2). For the 3'-OH unblocked analogs, dU.V and dU.VI, kpol rate constants were 37 ± 6 s−1 and 36 ± 7 s−1, respectively, which are ~5- to 7-fold slower than their natural nucleotide counterparts. The maximum rate constants for dA.VI, dC.VI and dG.VI

### Table 1. PEP assay results using Therminator DNA polymerase

| Nucleotide          | IC50 (nM) | Incorporation bias |
|---------------------|-----------|-------------------|
| TTP                 | 1.9 ± 0.1 | N/A               |
| HOMedUTP            | 1.7 ± 0.1 | N/A               |
| dU.V                | 4.9 ± 0.2 | 2.9               |
| dU.VI               | 7.3 ± 0.6 | 4.3               |
| 3'-O-aminotTP       | 17.1 ± 1.7| 9.0               |
| 3'-O-azidomethyl-TP | No Incorp | N/A               |
| C2′-HOMedATP        | 1.0 ± 0.1 | N/A               |
| dA.VI               | 3.5 ± 0.1 | 3.5               |
| HOMedCTP            | 1.5 ± 0.1 | N/A               |
| dC.VI               | 1.5 ± 0.1 | 1.0               |
| C2′-HOMedGTP        | 2.1 ± 0.1 | N/A               |
| dG.VI               | 2.8 ± 0.2 | 1.3               |

**IC50 data obtained from Litosh et al. (38).**

### Table 2. Pre-steady-state kinetic constants using Therminator DNA polymerase

| Nucleotide          | kpol (s−1) | KD (μM) | kpol/KD (μM−1 s−1) | Selection (analog/TTP) |
|---------------------|------------|---------|---------------------|------------------------|
| TTP                 | 170 ± 4    | 73 ± 3  | 2.3                 | 1.0                    |
| HOMedUTP            | 250 ± 11   | 33 ± 7  | 7.6                 | 3.3                    |
| dU.V                | 37 ± 6     | 15 ± 2  | 2.5                 | 1.1                    |
| dA.VI               | 45 ± 1     | 4.0 ± 0.1| 11                  | ND                     |
| dC.VI               | 36 ± 1     | 8.7 ± 1.1| 4.1                 | ND                     |
| dG.VI               | 35 ± 1     | 16 ± 3  | 2.2                 | ND                     |
| dU.VI               | 36 ± 7     | 12 ± 1  | 3.0                 | 1.3                    |

The nucleotide concentration range used in these studies were 5–1000 μM for TTP, 25–1000 μM for HOMedUTP and 1–50 μM for dU.V, dA.VI, dC.VI, dG.VI and dU.VI. ND indicates that kinetic parameters for their corresponding natural nucleotides were not determined.
were very similar to that of their hydroxymethyluracil counterparts (Table 2). The binding constants ($K_D$) for dU.V and dU.VI were ~2 to 6-fold lower than those for TTP and HOMedUTP, suggesting higher binding affinities of the 3'-OH unblocked terminators within the Therminator active site. Similar ground state binding constants were observed for dA.VI, dC.VI and dG.VI (Table 2). Of the four Lightning Terminators, dA.VI showed the fastest $k_{pol}$ rate constant and lowest $K_D$ binding constant of the nucleotide set.

The pre-steady-state kinetic parameter ($k_{pol}/K_D$) can be used to express the catalytic efficiency of a given nucleotide or nucleotide analog (47). The catalytic efficiencies of the Lightning Terminators$^\text{TM}$ ranged from 2.2 to 11 $\mu$M$^{-1}$ s$^{-1}$ similar to those of TTP and HOMedUTP, Table 2. These data provide good evidence that the 3'-OH unblocked hydroxymethyluracil terminators are as efficient in catalytic performance as the natural nucleotide TTP and strongly suggest that the other Lightning Terminators$^\text{TM}$ would show similar catalytic performance with their corresponding native nucleotides.

**Lightning Terminators$^\text{TM}$ exhibit fast burst kinetics**

To further characterize the enzymatic performance of 3'-OH unblocked terminators, burst kinetic experiments were performed with the primer/template duplex in excess of Therminator DNA polymerase. For natural nucleotides, previous burst studies have shown biphasic kinetics of an initial fast turnover rate, called the burst rate ($k_{burst}$), occurring at the active site of polymerase, followed by a slower steady-state rate ($k_{SS}$) that is dependent on the duplex dissociating from polymerase. Therminator DNA polymerase displayed the biphasic burst pattern similar to those for Klenow fragment (52), AmpliTaq CS (53), RB69 (54), Vent(exo$^-$) and Vent(exo$^-$) A488L (45), Sso PolB(exo$^-$) (55) and Pfu(exo$^-$) (56) DNA polymerases. The rapid burst rates ($k_{burst}$) were 146 ± 60 s$^{-1}$ and 130 ± 9 s$^{-1}$ followed by slow steady-state turnover rates ($k_{SS}$) of 0.03 ± 0.01 s$^{-1}$ and 0.07 ± 0.01 s$^{-1}$ for TTP and HOMedUTP, respectively; see Supplementary Figure S1A and B. The burst amplitude ($A$) was measured from these curves, indicating that ~90% of the Therminator DNA polymerase preparation was active. Remarkably, the Lightning Terminators dA.VI, dC.VI, dG.VI and dU.VI (Figure 2) and dU.V (Supplementary Figures S1C) also displayed a biphasic burst pattern similar to that for natural nucleotides. The rapid burst rates ($k_{burst}$) ranged from 24 ± 9 s$^{-1}$ to 51 ± 30 s$^{-1}$ followed by slow steady-state turnover rates ($k_{SS}$) that ranged from 0.02 ± 0.01 s$^{-1}$ to 0.06 ± 0.01 s$^{-1}$ (Table 3).

**Slow mismatch incorporation rate governs improved nucleotide selectivity for dU.V and dU.VI**

To maintain replication fidelity, DNA polymerases have evolved mechanisms for exquisite selectivity to insert the correct nucleotide across its complementary templating...
Nucleotide Template base $k_{\text{pol}}$ (s$^{-1}$) $K_D$ ($\mu$M) $k_{\text{pol}}/K_D$ ($\mu$M$^{-1}$ s$^{-1}$) Nucleotide selectivity

TTP

C 72 ± 1 150 ± 8 0.48 4.9 11
G 55 ± 2 340 ± 50 0.16 14 22
T 97 ± 4 290 ± 19 0.33 7.0 44

dU.V

C 0.045 ± 0.035 12 ± 2 3.8 × 10$^{-3}$ 630 1300
G 0.030 ± 0.002 25 ± 1 1.2 × 10$^{-3}$ 2000 740
T 0.053 ± 0.011 45 ± 4 1.2 × 10$^{-3}$ 2000 850

dU.VI

C 0.063 ± 0.020 13 ± 3 4.8 × 10$^{-3}$ 620 590
G 0.048 ± 0.016 44 ± 6 1.1 × 10$^{-3}$ 2800 400
T 0.035 ± 0.010 34 ± 10 1.0 × 10$^{-3}$ 2900 540

$^a$IC$_{50}$ data can be found in Litosh et al. (38).

$^b$IC$_{50}$ data can be found in Table 4.

Corr., correct template base; MisM, mismatched template base.
dU.VI is a significant reduction in the maximum incorporation rate ($k_{\text{pol}}$).

**Tert-butyl-2-nitrobenzyl modified HOMedUTP analogs exhibited single-base termination in homopolymer repeats**

A challenge in using 3'-OH unblocked terminators is creating appropriate modification(s) to the terminating group so that DNA synthesis is stopped after a single-base addition. As the 3'-OH group is the appropriate substrate, incorporating the next incoming nucleotide or nucleotide analog is possible with the effect of reading through the target templating base and causing type I dephasing (i.e. the primer has advanced beyond the target templating base) of the primary signal (30). If observed, the rate $k_{\text{pol}(+2)}$ for the second base incorporation can be measured to determine the extent of the nucleotide read-through. For example, Bowers et al. described pre-steady-state kinetics employing two-base homopolymer templates, for which $k_{\text{pol}(+2)}$ rates were measured for all of their 3'-OH unblocked ‘virtual’ terminators (57).

We recently reported that the size of the $\alpha$-substitution group plays an important role in ‘tuning’ the termination properties of 2-nitrobenzyl alkylated HOMedUTP analogs (38). Therefore, we performed pre-steady-state kinetic experiments using a two-base ‘AA’ homopolymer template for dU.V and dU.VI. Unlike that of Bowers et al. who conducted their termination experiments at submicromolar nucleotide concentrations (i.e. from 100 to 250 nM), termination assays were performed at 10 μM over the time course of 0.5 to 20 min. Both dU.V and dU.VI (Figure 3) were rapidly incorporated at the first base position (100% by 2 min) and then terminated DNA synthesis at that position. We could not detect any appreciable signal at the expected second-base position up to incubation times of 20 min. Single-base termination was verified by gel electrophoresis, which showed that the DNA products of photochemical cleavage comigrated with that of a singly incorporated TTP nucleotide (compare lanes Ct and Pc). Thus, we were unable to measure $k_{\text{pol}(+2)}$ rates for either analog, indicating that dU.V and dU.VI are efficient at single-base termination for homopolymer template sequences.

**DISCUSSION**

In this study, we performed a direct comparison of 3'-OH unblocked and 3'-O-modified terminators using Therminator DNA polymerase, previously shown to incorporate a variety of sugar-modified (32,41,49,51) and base-modified (37,38) nucleotides. Incorporation biases of modified nucleotides appear to be governed more by the site to which the terminating group is attached rather than its molecular size, as both 3'-OH unblocked nucleotides (dU.V and dU.VI) having a much larger terminating group showed less bias than 3'-O-aminomethyl-TTP. A key advantage of the 3'-OH unblocked Lightning Terminators™ is their fast nucleotide-incorporation rates. Remarkably, the Lightning Terminator™ analogs also exhibited a characteristic biphasic burst pattern, which is not observed with many modified nucleotide substrates (45,53). We attribute this superior performance in enzyme kinetics primarily to having an unblocked 3'-OH group. To further highlight this point, we characterized 3'-O-azidomethyl-TTP with Terminator III DNA polymerase, which contains numerous variants in the steric gate region: L408S, Y409A and P410V. Unlike the results for Therminator, 3'-O-azidomethyl-TTP was incorporated by Terminator III. Pre-steady-state kinetic experiments revealed a maximum rate of incorporation ($k_{\text{pol}}$) to be 0.68 ± 0.04 s⁻¹ and the nucleotide ground state binding constant ($K_D$) to be 7.1 ± 0.1 μM. Thus, even under optimized conditions

![Figure 3. Single-base termination of dU.V and dU.VI. Therminator DNA polymerase was bound to primer/template complex in 1x Thermopol buffer and subjected to 10 min incubation at 75°C in HPLC water (lane 'P' or primer), 50 nM TTP (lane ‘Ct’ or control) or 10 μM dU.V followed by a 90 s exposure to 365 nm UV light (lane ‘Pc’ or photochemical cleavage)—control lanes demonstrating that the incorporation bands of dU.V and dU.VI are the result of a single-base incorporation event. The remaining lanes represent incubation time courses of 10 μM dU.V or 10 μM dU.VI. Prior to performing the termination assays, dU.V and dU.VI were purified using the mop-up assay to remove trace amounts of HOMedUTP (43). Weighted-sum analysis was performed, yielding a value $= 1.0$ (white asterisks) at time points from 2 to 20 min (38). We note that the weighted-sum method, which has been reported previously (38), is used to measure quantitatively the termination property of nucleotide analogs being extended along a homopolymer stretch of complementary template bases. The slightly slower mobility of dU.VI termination products over those of dU.V results from the higher mass of the OMe group. Assays were performed in triplicate, a representative gel of which is shown.](https://example.com/figure3.png)
involving the relaxation of the steric gate residues, the incorporation rates for the Lightning Terminators™ were still faster by >50-fold.

Crystal structures of the apo enzyme for many Family B polymerases have been determined including 9N-7 (58), D. Tok (59), KOD1 (60), Pfu (61), RB69 (62), Sso PolB1 (63) and Tgo (64). As the tertiary structure of RB69 bound with a primer/template duplex and TTP has also been solved (65), these amino acid coordinates can serve as a model for other hyperthermophilic DNA polymerases, including Therminator. For example, the 2-deoxyribose group of the incoming TTP stacks on top of the phenolic ring of a conserved tyrosine group at amino acid position 416 (Y416), corresponding to the Y409 residue in Therminator and DNA polymerase. The 3'-OH group of TTP is positioned to make hydrogen bonds to the –NH group of the peptide bond for Y409 and to its non-bridging oxygen of the β-phosphate group. Figure 4A illustrates a model whereby the 3'-OH group of dU.VI is hydrogen bonding with these groups. This model is supported by kinetic data showing that the absence of 3'-OH group for nucleotide terminators, such as 2',3'-ddNTPs, disrupts hydrogen bonding to these functional groups and lowers k_{pol} by 400- to 1100-fold in Vent(exo⁻) and RB69 DNA polymerases, respectively (45). Figure 4B illustrates a model whereby 3'-O-azidomethyl-TTP disrupts hydrogen binding and the 3'-O-modification causes steric clashing with one or more amino acid residues within the active site, providing an explanation for its non-incorporation by Therminator polymerase.

In the present study, k_{pol} constants for dU.V and dU.VI were only slightly lower (5- and 7-fold) than TTP or HOMedUTP, respectively and dA.VI, dC.VI and dG.VI showed similar rate constants to dU.V and dU.VI. These data suggest that the (S)-tert-butylnitrobenzyl group has only a modest adverse effect on the kinetic properties of these reversible terminators.

Lightning Terminators™ dU.V and dU.VI show higher nucleotide binding affinities (i.e. lower $K_D$ constants) than TTP for both correct (Table 2) and mismatched (Table 5) base-pairs. This finding suggests that the hydrophobic (S)-tert-butylnitrobenzyl group may bind favorably within the active site of Therminator DNA polymerase, independent of the templating base. We note that the fold differences, however, in ground state nucleotide binding constants for correct and mismatched base-pairs were similar for TTP (range 2.1- to 4.7-fold), dU.V (range 0.8- to 2.8-fold) and dU.VI (range 1.1- to 3.7-fold). These data suggest that Therminator DNA polymerase is a weak discriminator for nucleotide binding of mismatched base-pairs that is independent of the substrates tested here. Despite the overall structural similarities, Joyce and Benkovic provide good evidence that even members of the same DNA polymerase family show extreme variability in the ‘kinetic checkpoint’ of weak versus strong discrimination for ground state binding (24).

Figure 4. Models of (A) dU.VI and (B) 3'-O-azidomethyl-TTP interactions with Therminator DNA polymerase. The RB69 DNA polymerase ternary structure [PDB ID: 1IG9; see reference (65)] is used as a structural model for Therminator DNA polymerase and its active site [from 9N-7 DNA polymerase (58)] was aligned with bound TTP using the pair fit function in MacPyMol (The PyMOL Molecular Graphics System, Version 1.5.0.1 Schrödinger, LLC). (A) dU.VI and (B) 3'-O-azidomethyl-TTP were drawn as extension of the three-dimensional structural model for TTP. Dotted lines in (A) represent hydrogen bonds between the 3'-OH group of dU.VI and its non-bridging oxygen of the β-phosphate group and –NH group of the peptide bond for Y409 (Therminator DNA polymerase amino acid numbering) that illustrate proper geometric alignment of these groups for efficient catalysis. The extension of the 3'-O-azidomethyl group in (B) is expected to cause a steric clash with the phenolic group side chain of Y409, thereby abolishing its incorporation efficiency. Improved incorporation performance with Therminator III, which contains the three substitutions (L408S, Y409A and P410V) that reduce the size of each amino acid side chain, supports this model.
efficiency. We cannot, however, formally rule out that the chemistry step \(k_j \) becomes rate limiting for the Lightning Terminators\textsuperscript{TM} dU.V and dU.VI. In this alternative model, Therminator DNA polymerase undergoes a conformational change into the closed form, but the overall geometric shape of the mismatched base-pairs induce the reactive groups to be out of alignment within the active site, thus hindering the phosphoryl transfer step. Further kinetic studies are required to resolve these two models.

As noted above, the challenge of any 3'-OH unblocked terminator is the availability of this hydroxyl group for the next incoming nucleotide. Nonetheless, we present data that the common (S)-tert-butyl group attached to the \(\alpha\)-carbon of the 2-nitrobenzyl group for dU.V and dU.VI causes absolute single-base termination (Figure 3). Because the 3'-OH is unblocked, termination of DNA synthesis is presumed to occur by a mechanism different than that of 3'-modified analogs, such as 2',3'-ddNTPs or 3'-O-modified reversible terminators. At least two models can be considered to describe the modes of action that may cause DNA polymerases to terminate DNA synthesis with 3'-OH unblocked terminators: (i) misalignment model and (ii) translocation-defective model. In the misalignment model, the incoming Lightning Terminator is incorporated into the primer strand followed by a translocation step that allows the next incoming nucleotide to bind at the N-site (i.e. the nucleotide binding site). If the 3'-OH group of dU.V or dU.VI nucleotide is not properly aligned with the incoming nucleotide bound within the closed active site, then phosphoryl transfer cannot occur and synthesis halts. The translocation-defective model of termination proposes that the incoming dU.V or dU.VI is incorporated, but translocation is blocked, leaving the 3'-terminal nucleotide in the pre-translocation P-site (i.e. the primer binding site). Failure of the next incoming nucleotide to bind within the pre-translocation site results in a termination event. Previous studies by Michailidis and colleagues have demonstrated that the 3'-OH unblocked, sugar-modified nucleotide 4'-ethynyl-2-fluoro-dATP blocks human immunodeficiency virus (HIV) RT translocation and supports the translocation-defective model for termination (66). Further experimentation will be required to determine the mechanism(s) by which dU.V and dU.VI terminate DNA synthesis. Understanding how 3'-OH unblocked, base-modified nucleotides terminate synthesis could lead to new insights into polymerase mechanisms for discrimination and active site architecture and may lead to the development of a new class of effective nucleotide inhibitors as antiviral therapeutic agents.

We believe that the superior enzymatic performance properties of Lightning Terminators\textsuperscript{TM} will have the potential to improve the performance of NGS technologies in speed, cost and accuracy. We have demonstrated that the Lightning Terminators\textsuperscript{TM} exhibit fast nucleotide-incorporation rates, which should result in shorter cycle times with the CRT method (29,30). These fast-acting 3'-OH unblocked terminators are comparable in speed performance to the Pacific Biosciences ‘real-time’ nucleotides, considered to be one of the fastest chemistries currently used in NGS technologies (67,68). A major difference between these nucleotide reagents is accuracy, as the Pacific Biosciences system has been shown to have one of the highest error rates of all NGS technologies (68,69). The Illumina platform, which employs the 3'-O-azidomethyl terminator chemistry (34), also shows high error rates that have been attributed, at least in part, to chemistry-specific systematic errors (70). A direct comparison between Illumina and Complete Genomics (71) technologies in high coverage sequencing (~76× each platform) of the same human genome found that of the ~3.7 million single nucleotide variants called, 88% were concordant between platforms, with platform-specific calls having a high false-positive rate of at least 35% (72). Here, we have demonstrated that the Lightning Terminators\textsuperscript{TM} dU.V and dU.VI show high fidelity in DNA synthesis compared with natural nucleotides. This enzymatic property should translate directly into improved accuracy of primary DNA sequence data. While detailed cost analysis has not been described here, we anticipate that these fast-acting, high-performance, 3'-OH unblocked Lightning Terminators\textsuperscript{TM} will result in lower reagent usage, thus reducing a major cost component of NGS technologies. Having now developed a breadboard instrument (73), experiments are underway to demonstrate these benefits by sequencing the E. coli genome using the complete set of dye-labeled Lightning Terminators\textsuperscript{TM}.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online: Supplementary Figure 1.

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