A Non-natural Nucleoside with Combined Therapeutic and Diagnostic Activities against Leukemia

Edward A. Motea,† Irene Lee,‡ and Anthony J. Berdis§,†

ABSTRACT: Acute lymphoblastic leukemia (ALL) is the most common type of childhood cancer, presenting with approximately 5,000 new cases each year in the United States. An interesting enzyme implicated in this disease is terminal deoxynucleotidyl transferase (TdT), a specialized DNA polymerase involved in V(D)J recombination. TdT is an excellent biomarker for ALL as it is overexpressed in ~90% of ALL patients, and these higher levels correlate with a poor prognosis. These collective features make TdT an attractive target to design new selective anti-cancer agents against ALL. In this report, we evaluate the anti-leukemia activities of two non-natural nucleotides designated 5-nitroindolyl-2′-deoxynucleoside triphosphate (5-NITP) and 3-ethyl-5-nitroindolyl-2′-deoxynucleoside triphosphate (3-Eth-5-NITP). Using purified TdT, we demonstrate that both non-natural nucleotides are efficiently utilized as TdT substrates. However, 3-Eth-5-NITP is poorly elongated, and this observation validates its activity as a chain-terminator for blunt-end DNA synthesis. Cell-based experiments validate that the corresponding non-natural nucleoside produces robust cytostatic and cytotoxic effects against leukemia cells that overexpress TdT.

Cell replication is the process by which genetic information is duplicated to produce two identical copies of an organism’s genome.1 This complex biological process typically involves a conglomerate of different proteins working in an orderly fashion.2,3 However, the most important enzyme involved in this process is terminal deoxynucleotidyl transferase (TdT), a specialized DNA polymerase involved in V(D)J recombination. TdT is also noteworthy for its role in cancer as it is implicated in this disease.12,13 Furthermore, higher levels of TdT activity correlate with a poor prognosis as lower remission rates are observed in patients with TdT-positive leukemia compared to those with normal TdT levels.14 Finally, ALL is the most common form of childhood cancer in the United States, presenting with approximately 5,000 new cases each year.15 If left untreated, ALL typically causes death within a few months.16

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These collective features have prompted efforts to develop selective inhibitors of TdT as anti-cancer agents against TdT-positive leukemias. One noteworthy example is the nucleoside analogue cordycepin (3′-deoxyadenosine) (Figure 1A) that lacks a 3′-OH and thus terminates DNA synthesis after its incorporation into DNA. This analogue produces cytotoxic effects against TdT-positive leukemia, especially when combined with the adenosine deaminase inhibitor, deoxycoformycin.\(^{17,18}\) Unfortunately, cordycepin is not a truly selective TdT inhibitor as it is also utilized by template-dependent DNA polymerases involved in chromosomal DNA synthesis.\(^ {19} \) The ability of these polymerases to incorporate but not elongate cordycepin terminates DNA synthesis in both cancerous and healthy cells. This non-selective killing can cause adverse side effects such as immunosuppression, fatigue, nausea, vomiting, and alopecia.\(^ {20} \)

Template-independent synthesis catalyzed by TdT is mechanistically similar to the replication of non-instructional DNA lesions such as abasic sites. We have studied the molecular mechanism for how abasic sites are replicated using non-natural nucleotides that are incorporated with variable efficiencies.\(^ {21−25}\) One exceptional analogue is 5-nitroindolyl-2′-deoxyriboside triphosphate (5-NITP) (Figure 1A) as it displays an incredibly high catalytic efficiency of \(10^6 \text{ M}^{-1} \text{ s}^{-1}\) for insertion opposite this lesion.\(^ {21}\) This value is \(\sim 1,000\)-fold higher than that for dATP, the preferred natural nucleotide substrate.\(^ {26}\) Although 5-NI can pair equally with all four natural nucleobases,\(^ {27,28}\) we demonstrated that the non-natural nucleotide, 5-NITP, is inserted poorly opposite undamaged DNA with low catalytic efficiencies of \(\sim 10^3 \text{ M}^{-1} \text{ s}^{-1}\).\(^ {21}\) Finally, we recently described a synthetic protocol to convert 5-NITP into a chemical probe that can visualize and quantify the replication of non-instructional abasic sites under \textit{in vitro} conditions.

Figure 1. (A) Chemical structures for cordycepin (3′-deoxyadenosine), 2′-deoxyadenosine, 5-nitroindolyl-2′-deoxyribose, and 3-ethynyl-5-nitroindolyl-2′-deoxyribose. (B) Strategy for using “clickable” nucleotides to monitor template-independent DNA synthesis. TdT is designated in pink.
conditions. In this case, placement of an ethynyl moiety at the 3-position of the non-natural nucleotide allows the analogue to be tagged with a fluorogenic probe via click chemistry once it is incorporated opposite the DNA lesion (Figure 1B). In this report, we describe the ability of 3-ethynyl-5-nitroindolyl-2′-deoxynucleoside triphosphate (3-Eth-5-NITP) to function as an efficient and potent chain-terminating nucleotide for TdT. In addition, we demonstrate that the corresponding nucleoside, 3-ethynyl-5-nitroindolyl-2′-deoxynucleoside (3-Eth-5-NIdR), functions as a novel theranostic anti-cancer agent against leukemia cells that overexpress TdT. The unique activities of this non-natural nucleoside against ALL highlights the selective...
inhibition of TdT activity. Potential clinical applications of this novel nucleoside analogue are discussed.

## RESULTS AND DISCUSSION

**Utilization of Non-natural Nucleotides by TdT.** The biological function of TdT is to expand immunological diversity by randomly incorporating dNTPs into single-stranded DNA during V(D)J recombination. In vitro studies with purified TdT have demonstrated that while the polymerase utilizes all natural dNTPs, there is a bias for incorporating dGTP and dTTP versus dATP and dCTP. TdT also utilizes nucleotide analogues including 2′,3′-dideoxy nucleotides, dinucleoside 5′,5′-tetraphosphates, and intrinsically fluorescent nucleotide analogues. In this report, we tested if TdT also incorporates indoly-2′-deoxyribose-5′-triphosphates that bear non-natural moieties such as ethyl and nitro groups at the 3′- and 5′-position, respectively, of the indole base. Polymerization reactions were performed as outlined in Figure 2A in which 100 μM of natural (dTTP and dGTP) or non-natural (5-NITP or 3-Eth-5-NITP) nucleotides were added to a solution containing 6 units of TdT preincubated with 1.5 μM single-stranded DNA substrate (14-mer). Aliquots of the reaction were quenched with EDTA at variable time points, and the polymerization reactions were then subjected to denaturing polyacrylamide gel electrophoresis to separate extended primers from unreacted substrate. Representative data provided in Figure 2B shows that both 5-NITP and 3-Eth-5-NITP are utilized by TdT as efficiently as the natural purines, dATP and dGTP (Figure 2B). In contrast, 3-Eth-5-NITP is poorly terminated (Figure 2B), indicating that it behaves as a universal chain terminator of DNA synthesis. Indeed, extension beyond 3′-3-Eth-5-NITP is not observed, even after longer reaction times of up to 20 min (Supplemental Figure 2).

The ability of 3′-3-Eth-5-NITP to terminate DNA synthesis catalyzed by TdT was further evaluated through a series of competition experiments. TdT was preincubated with single-stranded DNA substrate and then mixed with a solution containing 10 μM dNTPs in the absence and presence of variable concentrations of 3′-3-Eth-5-NITP (0.5–50 μM). Reactions were terminated after 3 min by adding 200 mM EDTA, and the replication products were separated via denaturing gel electrophoresis. Representative data provided in Figure 2E shows that in the absence of 3′-3-Eth-5-NITP, TdT randomly incorporates dNTPs to generate replication products with lengths ranging from DNA_{14-1} to DNA_{14+}. As the concentration of 3′-3-Eth-5-NITP is increased, there is a concomitant decrease in the amount of replication products greater than DNA_{14+}. Note that under these conditions, products corresponding to DNA_{14+} accumulate since 3′-3-Eth-5-NITP is a non-extendable nucleotide substrate for TdT (vide supra). As such, the percent TdT activity as a function of 3′-3-Eth-5-NITP concentration was plotted (Figure 2F), and the resulting sigmoidal curve was fit to eq 3 to yield an IC_{50} value of 3.9 ± 1.0 μM for 3′-3-Eth-5-NITP. A true K_{i} value for 3′-3-Eth-5-NITP of 0.51 ± 0.13 μM was obtained using the Cheng-Prusoff equation (eq 4) to normalize this IC_{50} value for the concentration of dNTPs and their corresponding K_{i} values. The calculated K_{i} value of 0.51 μM for 3′-3-Eth-5-NITP is in good agreement with the K_{i} value of 0.19 μM measured through initial velocity studies (Table 1). Collectively, these data indicate that 3′-3-Eth-5-NITP is a bona fide chain-terminating substrate for TdT, even in the presence of physiological concentrations of dNTPs.

### Defining the Potency of Non-natural Nucleosides against ALL Cell Lines.

The facile utilization and chain termination capabilities of 3′-3-Eth-5-NITP against TdT suggest that it may have therapeutic potential against ALL. This hypothesis was tested by measuring the cytotoxic and/or cytostatic effects of the corresponding nucleoside, 3′-3-Eth-5-NITR, and 5-nitroindolyl-2′-deoxynucleoside (5-NITR) against various ALL cell lines including MOLT4, CEM-C7, Jurkat, RS4(11), JS45.01, and Loucy. Western blot analyses was first performed to determine TdT levels in each ALL cell line. As illustrated in Figure 3A, each cell line contains variable levels of TdT, ranging from none (Loucy) to low (Jurkat, RS4(11)) to high (MOLT4, JS45.01, and CEM-C7). We next tested the efficacy of 5-NITR and 3′-3-Eth-5-NITR by treating exponentially growing cells with variable concentrations (0.1–100 μg mL\(^{-1}\)) of each non-natural nucleoside for time periods of up to 3 days. Initial experiments used the MOLT4 cell line since this ALL cell line displays resistance to various chemotherapeutic agents.

Table 1. Summary of Kinetic Parameters for the Incorporation of Natural and Non-natural Nucleotides by Terminal Deoxynucleotidyl Transferase

<table>
<thead>
<tr>
<th>dNTP</th>
<th>K_{i} (μM)</th>
<th>V_{max} (nM s(^{-1}))</th>
<th>V_{max}/K_{i} (s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>dATP</td>
<td>1.5 ± 0.4</td>
<td>3.3 ± 0.3</td>
<td>0.0022 ± 0.0005</td>
</tr>
<tr>
<td>dGTP</td>
<td>8.0 ± 0.9</td>
<td>29.2 ± 1.1</td>
<td>0.0037 ± 0.0005</td>
</tr>
<tr>
<td>5-NITP</td>
<td>4.6 ± 0.6</td>
<td>11.5 ± 0.4</td>
<td>0.0025 ± 0.0003</td>
</tr>
<tr>
<td>3′-3-Eth-5-NITP</td>
<td>0.19 ± 0.04</td>
<td>3.2 ± 0.1</td>
<td>0.0168 ± 0.0004</td>
</tr>
</tbody>
</table>

Importantly, the catalytic efficiency for 3′-3-Eth-5-NITP is 7-fold higher than that for 5-NITP. This enhancement is caused by a 24-fold decrease in the K_{i} for 3′-3-Eth-5-NITP that offsets a 3.5-fold decrease in V_{max}. This result is noteworthy as similar effects on K_{i} were observed for incorporating 3′-3-Eth-5-NITP opposite an abasic site. These results highlight the universal requirement of nucleobase hydrophobicity and π-electron interactions during template-independent DNA synthesis. However, one important distinction is that TdT extends beyond 5-NITP with an overall efficiency that rivals that of the natural purines, dATP and dGTP (Figure 2B). In contrast, 3′-3-Eth-5-NITP is poorly extended (Figure 2B), indicating that it behaves as a universal chain terminator of DNA synthesis. Indeed, extension beyond 3′-3-Eth-5-NITP is not observed, even after longer reaction times of up to 20 min (Supplemental Figure 2).

The ability of 3′-3-Eth-5-NITP to terminate DNA synthesis catalyzed by TdT was further evaluated through a series of competition experiments. TdT was preincubated with single-stranded DNA substrate and then mixed with a solution containing 10 μM dNTPs in the absence and presence of variable concentrations of 3′-3-Eth-5-NITP (0.5–50 μM). Reactions were terminated after 3 min by adding 200 mM EDTA, and the replication products were separated via denaturing gel electrophoresis. Representative data provided in Figure 2E shows that in the absence of 3′-3-Eth-5-NITP, TdT randomly incorporates dNTPs to generate replication products with lengths ranging from DNA_{14-1} to DNA_{14+}. As the concentration of 3′-3-Eth-5-NITP is increased, there is a concomitant decrease in the amount of replication products greater than DNA_{14+}. Note that under these conditions, products corresponding to DNA_{14+} accumulate since 3′-3-Eth-5-NITP is a non-extendable nucleotide substrate for TdT (vide supra). As such, the percent TdT activity as a function of 3′-3-Eth-5-NITP concentration was plotted (Figure 2F), and the resulting sigmoidal curve was fit to eq 3 to yield an IC_{50} value of 3.9 ± 1.0 μM for 3′-3-Eth-5-NITP. A true K_{i} value for 3′-3-Eth-5-NITP of 0.51 ± 0.13 μM was obtained using the Cheng-Prusoff equation (eq 4) to normalize this IC_{50} value for the concentration of dNTPs and their corresponding K_{i} values. The calculated K_{i} value of 0.51 μM for 3′-3-Eth-5-NITP is in good agreement with the K_{i} value of 0.19 μM measured through initial velocity studies (Table 1). Collectively, these data indicate that 3′-3-Eth-5-NITP is a bona fide chain-terminating substrate for TdT, even in the presence of physiological concentrations of dNTPs.

**Defining the Potency of Non-natural Nucleosides against ALL Cell Lines.** The facile utilization and chain termination capabilities of 3′-3-Eth-5-NITP against TdT suggest that it may have therapeutic potential against ALL. This hypothesis was tested by measuring the cytotoxic and/or cytostatic effects of the corresponding nucleoside, 3′-3-Eth-5-NITR, and 5-nitroindolyl-2′-deoxynucleoside (5-NITR) against various ALL cell lines including MOLT4, CEM-C7, Jurkat, RS4(11), JS45.01, and Loucy. Western blot analyses was first performed to determine TdT levels in each ALL cell line. As illustrated in Figure 3A, each cell line contains variable levels of TdT, ranging from none (Loucy) to low (Jurkat, RS4(11)) to high (MOLT4, JS45.01, and CEM-C7). We next tested the efficacy of 5-NITR and 3′-3-Eth-5-NITR by treating exponentially growing cells with variable concentrations (0.1–100 μg mL\(^{-1}\)) of each non-natural nucleoside for time periods of up to 3 days. Initial experiments used the MOLT4 cell line since this ALL cell line displays resistance to various chemotherapeutic agents.

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due to higher levels of TdT. Figure 3B provides representative time courses for the number of viable (left) and non-viable (right) MOLT4 cells in the absence and presence of non-natural nucleosides. (C) Time courses in the number of viable (left) and non-viable (right) Loucy cells in the absence and presence of non-natural nucleosides. (D) Plot correlating IC₅₀ values of 3-Eth-5-NIdR against various ALL cell lines versus the cellular content of TdT in each cell line. (E) Plot correlating LD₅₀ values of 3-Eth-5-NIdR against various ALL cell lines versus the respective cellular content of TdT in each ALL cell line. (F) Dual parameter flow cytometry of MOLT4 cells treated with (i) DMSO, (ii) 100 μg mL⁻¹ 5-NIdR, (iii) 10 μg mL⁻¹ 3-Eth-5-NIdR, and (iv) 40 μg mL⁻¹ 3-Eth-5-NIdR. Q1 represents cells that are necrotic. Q2 represents late-apoptotic cells. Q3 represents viable cells. Q4 represents early apoptotic cells.

Figure 3. Correlating the anti-cancer effects of non-natural nucleosides with cellular levels of TdT. (A) Western blot analyses examining TdT content in ALL cell lines including MOLT4, Jurkat, RS4(11), JS45.01, CEM-C7, and Loucy. TdT content was normalized against cellular levels of β-actin in each respective cell line. (B) Time courses in the number of viable (left) and non-viable (right) MOLT4 cells in the absence and presence of non-natural nucleosides. (C) Time courses in the number of viable (left) and non-viable (right) Loucy cells in the absence and presence of non-natural nucleosides. (D) Plot correlating IC₅₀ values of 3-Eth-5-NIdR against various ALL cell lines versus the cellular content of TdT in each cell line. (E) Plot correlating LD₅₀ values of 3-Eth-5-NIdR against various ALL cell lines versus the respective cellular content of TdT in each ALL cell line. (F) Dual parameter flow cytometry of MOLT4 cells treated with (i) DMSO, (ii) 100 μg mL⁻¹ 5-NIdR, (iii) 10 μg mL⁻¹ 3-Eth-5-NIdR, and (iv) 40 μg mL⁻¹ 3-Eth-5-NIdR. Q1 represents cells that are necrotic. Q2 represents late-apoptotic cells. Q3 represents viable cells. Q4 represents early apoptotic cells.

days post-treatment. In this case, the number of viable cells is reduced ~5-fold while there is a 2.5-fold increase in the number of non-viable cells. Analyses of the time courses in cell growth as a function of nucleoside concentration yield an IC₅₀ of 36.4 ± 5.8 μg mL⁻¹ and an LD₅₀ value of ~100 μg mL⁻¹ for 5-NIdR (Supplemental Figure 3). More striking effects are observed...
with 3-Eth-5-NIdR as cytostatic and cytotoxic effects are observed at a low concentration of 10 μg mL⁻¹ (Figure 3B). These effects are also dose-dependent as treatment with 40 μg mL⁻¹ shows a significant reduction in the number of viable cells coupled with a substantial increase in the number of non-viable cells (Figure 3B). Quantitative analyses of the data yield an IC₅₀ of 14.1 ± 2.4 μg mL⁻¹ and an LD₅₀ value 27.7 ± 1.7 μg mL⁻¹ for 3-Eth-5-NIdR (Supplemental Figure 3).

To interrogate if the cytostatic and cytotoxic effects of 5-NIdR and 3-Eth-5-NIdR are dependent upon the cellular level of TdT, we next measured their effects against the TdT-negative Loucy cell line (vide supra). Data provided in Figure 3C show that both non-natural nucleosides are significantly less potent against this TdT-negative leukemia cell line compared to the TdT-positive cell line, MOLT4. In particular, treatment with 100 μg mL⁻¹ of 5-NIdR produces only a weak cytostatic effect as the number of viable cells is reduced by <25%. In addition, treatment with 5-NIdR does not cause a substantial increase in the number of non-viable cells. 3-Eth-5-NIdR is also ineffective as significant cytostatic or cytotoxic effects are not observed at a concentration of 40 μg mL⁻¹.

Identical cell-based experiments were performed with the other ALL cell lines, and the corresponding IC₅₀ and LD₅₀ values for 5-NIdR and 3-Eth-5-NIdR against each cell line are summarized in Table 2. Inspection of these data indicates that 3-Eth-5-NIdR is more potent than the parental nucleoside, 5-NIdR. The increased potency of 3-Eth-5-NIdR likely reflects the higher catalytic efficiency for the corresponding nucleoside triphosphate to act as a chain-terminating substrate for TdT. Consistent with this mechanistic, the data also show that 3-Eth-5-NIdR displays higher potency against ALL cells that overexpress TdT compared to cells with lower TdT levels. This is best illustrated in Figure 3D, which shows a linear relationship between the IC₅₀ values of 3-Eth-5-NIdR in various ALL cell lines as a function of cellular TdT content in each respective cell line. In general, there is an excellent correlation (R² = 0.695, p < 0.0001) between the anti-cancer effects of the non-natural nucleoside with TdT level. Similar analyses were performed by plotting the LD₅₀ values of 3-Eth-5-NIdR as a function of TdT content (Figure 3E). These data also highlight a correlative effect between the cell killing effects of the non-natural nucleoside with the cellular content of the TdT, the proposed molecular target for the corresponding chain-terminating nucleotide substrate.

### Defining the Mechanism of Cell Death Induced by Non-natural Nucleosides

The mechanism by which these non-natural nucleosides induce cell death was interrogated using dual parameter FACS analyses measuring propidium iodide uptake and Alexa Fluor488 annexin V conjugate staining. This allows live cells (unstained with either fluorophore) to be distinguished from cells that are early apoptotic (annexin V staining only), late apoptotic (propidium iodide and annexin V staining), and necrotic (propidium iodide staining only). Representative data provided in Figure 3F shows that MOLT4 cells treated with 5-NIdR have higher levels of early and late stage apoptosis compared to cells treated with DMSO. In this case, treatment with 100 μg mL⁻¹ 5-NIdR causes a 5-fold increase in early and late stage apoptosis, respectively. Data summarized in Table 3 shows that the “clickable” nucleoside, 3-Eth-5-NIdR, is significantly more potent than treatment with 10 μg mL⁻¹ causes equivalent levels of apoptosis, while treatment with 40 μg mL⁻¹ results in 13- and 7-fold increases in early stage and late stage apoptosis, respectively. Furthermore, cells treated with either 5-NIdR or 3-Eth-5-NIdR show no significant PI uptake, thus indicating that neither non-natural nucleoside causes necrotic cell death. The induction of apoptosis was also independently confirmed by using a Agarose gel electrophoresis to observe DNA cleavage sites between nucleosomes that occur in chromatin at ~200-base pair intervals (Supplemental Figure 4).

### Diagnostic Activities of Non-natural Nucleosides

Cell-based experiments were performed to demonstrate that the “clickable” nucleoside, 3-Eth-5-NIdR, functions as a spectroscopic probe to measure the cellular activity of TdT. MOLT4 cells were treated with DMSO, EdU (a “clickable” thymidine analogue), 5-NIdR, and 3-Eth-5-NIdR for 2 days and then washed with PBS to remove any nucleoside and/or nucleotide not incorporated into DNA. After fixation and permeabilization, the cells were treated with AlexaFluor488-azide and Cu(I) catalyst to commence “clicking” of any incorporated non-natural nucleotide. Dual parameter flow cytometry applying PI staining coupled with detection of the AlexaFluor488 fluorophore was used to detect the “clicked” non-natural nucleotide in cellular DNA. As illustrated in Figure 4A, cells treated with DMSO have a low level (<0.5%) of AlexaFluor488 stained DNA, while cells treated with 10 μM EdU show significantly higher levels (36.1%). This high amount of “clicked” DNA reflects transport of EdU into the cell, conversion to the corresponding nucleoside triphosphate, and incorporation into the DNA of replicating cells. MOLT4 cells

#### Table 2. Summary of IC₅₀ and LD₅₀ of the Non-natural Nucleosides against Acute Lymphoblastic Leukemia Cell Lines

<table>
<thead>
<tr>
<th>nucleoside</th>
<th>cell line</th>
<th>IC₅₀ (μg mL⁻¹)</th>
<th>LD₅₀ (μg mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-NIdR</td>
<td>MOLT4</td>
<td>36 ± 6</td>
<td>~100</td>
</tr>
<tr>
<td>3-Eth-5-NIdR</td>
<td>MOLT4</td>
<td>14 ± 2</td>
<td>28 ± 4</td>
</tr>
<tr>
<td>5-NIdR</td>
<td>Loucy</td>
<td>&gt;&gt;100</td>
<td>&gt;&gt;100</td>
</tr>
<tr>
<td>3-Eth-5-NIdR</td>
<td>Loucy</td>
<td>62 ± 5</td>
<td>63 ± 3</td>
</tr>
<tr>
<td>5-NIdR</td>
<td>CEM-C7</td>
<td>~100</td>
<td>&gt;&gt;100</td>
</tr>
<tr>
<td>3-Eth-5-NIdR</td>
<td>CEM-C7</td>
<td>23 ± 2</td>
<td>~100</td>
</tr>
<tr>
<td>5-NIdR</td>
<td>Jurkat</td>
<td>~100</td>
<td>&gt;&gt;100</td>
</tr>
<tr>
<td>3-Eth-5-NIdR</td>
<td>Jurkat</td>
<td>49 ± 1</td>
<td>~100</td>
</tr>
<tr>
<td>5-NIdR</td>
<td>R84(11)</td>
<td>&gt;100</td>
<td>&gt;&gt;100</td>
</tr>
<tr>
<td>3-Eth-5-NIdR</td>
<td>R84(11)</td>
<td>23 ± 2</td>
<td>57 ± 2</td>
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<td>5-NIdR</td>
<td>J45.01</td>
<td>~100</td>
<td>&gt;&gt;100</td>
</tr>
<tr>
<td>3-Eth-5-NIdR</td>
<td>J45.01</td>
<td>34 ± 6</td>
<td>71 ± 6</td>
</tr>
</tbody>
</table>

#### Table 3. Summary of Viable, Apoptotic, and Necrotic MOLT4 Cells after Treatment with Non-natural Nucleosides

<table>
<thead>
<tr>
<th>condition</th>
<th>viable cells (%)</th>
<th>early apoptotic cells (%)</th>
<th>late apoptotic cells (%)</th>
<th>necrotic cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>899 ± 0.9</td>
<td>5.4 ± 0.1</td>
<td>3.5 ± 1.1</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>100 μg mL⁻¹</td>
<td>53.1 ± 6.7</td>
<td>26.3 ± 3.7</td>
<td>18.9 ± 3.4</td>
<td>1.5 ± 0.5</td>
</tr>
<tr>
<td>10 μg mL⁻¹</td>
<td>83.4 ± 2.7</td>
<td>10.5 ± 1.5</td>
<td>5.4 ± 1.0</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>40 μg mL⁻¹</td>
<td>63.3 ± 3.7</td>
<td>68.8 ± 0.9</td>
<td>23.7 ± 4.9</td>
<td>1.2 ± 0.2</td>
</tr>
</tbody>
</table>
treated with 10 μM EdU, 10 μg mL\(^{-1}\) 3-Eth-5-NIdR, 40 μg mL\(^{-1}\) 3-Eth-5-NIdR, and 100 μg mL\(^{-1}\) 5-NIdR. (B) Model for the ability of the non-natural nucleoside 3-Eth-5-NIdR to function as a theranostic agent against TdT-positive leukemia. (D) Structural comparison between conventional nucleoside analogues (cordycepin and clofarabine) with the non-natural nucleoside 3-Eth-5-NIdR.

Collectively, these data are consistent with the mechanism outlined in Figure 4C in which the non-natural nucleoside enters the cell via the activity nucleoside transporters, undergoes catabolism to the corresponding nucleoside triphosphate, and is then utilized by TdT. Once incorporated into DNA, the non-natural nucleotide inhibits cellular DNA replication and induces apoptosis. Finally, it should be emphasized that low levels (<0.5%) of AlexaFluor488 staining...
are detected in cells treated with 100 μg mL⁻¹ 5-NIdR. While this result could reflect a lack of stable incorporation of the non-natural nucleotide into DNA, it is more likely that the lack of the ethynyl moiety precludes covalent attachment of the azide-containing dye.

**Biological and Clinical Applications of Non-natural Nucleosides.** Acute lymphocytic leukemia (ALL) is the most common form of childhood cancer. As with all cancers, a fundamental feature of ALL is its hyperproliferative nature that is defined by uncontrollable and pro-mutagenic DNA replication. Nucleoside analogues are effective anti-cancer agents against leukemia as they produce anti-proliferative and/or cytotoxic effects by inhibiting DNA replication. Despite their widespread utility, however, most nucleoside analogues possess very narrow therapeutic windows that can create significant clinical problems. Indeed, the ability of conventional nucleoside analogues to non-selectively kill healthy cells causes severe side effects such as anemia, leukopenia, thrombocytopenia, and diarrhea/nausea that adversely influence a patient’s response to chemotherapy.

This problem is exacerbated since it is nearly impossible to rapidly and accurately assess the efficacy of conventional nucleoside analogues. Patient responses to chemotherapy are typically gauged by qualitative criteria ranging from the absence of overt disease symptoms to quantifying the ratio of normal versus cancerous blood cells. Clinical end points can take weeks or even months to accurately define. As such, the inability to assess drug action on shorter time scales (hours or days) significantly hinders physicians from making informed decisions regarding optimal dosing regimens. Our studies here demonstrate a pragmatic solution to overcome these complications by using 3-Eth-5-NIdR as a theranostic agent to treat leukemia. The theranostic potential of 3-Eth-5-NIdR is evident as it produces robust and potent therapeutic effects against TdT-positive leukemia cells while also possessing diagnostic properties to monitor its self-efficacy. The sections below elaborate on the properties of this unique theranostic agent, specifically comparing and contrasting its novel characteristics with nucleoside analogues currently used to treat leukemia.

Although conventional nucleoside analogues such as fludarabine and gemcitabine influence DNA synthesis on multiple levels, their primary effect is to directly inhibit DNA synthesis via incorporation and chain-termination by the corresponding nucleoside triphosphate. In these cases, a template-dependent polymerase inserts the analogue into DNA as efficiently as its natural counterpart. However, the absence of a usable 3'-OH group prematurely terminates DNA synthesis by creating a nucleic acid substrate that is refractory to elongation. The inability to complete DNA synthesis in a timely fashion stalls replication fork progression and activates apoptosis to cause cell death. The in vitro studies described here clearly show that 3-Eth-5-NITP is a potent chain-terminator for template-independent synthesis catalyzed by TdT. Indeed, the measured $K_{\text{m}}$ value of 190 nM for 3-Eth-5-NITP is ∼10-fold lower than that for natural purine nucleotides such as dATP. In addition, 3-Eth-5-NITP is highly selective for template-independent DNA synthesis catalyzed by TdT since it is poorly incorporated opposite normal templating bases. These activities alone represent important features required for a potential therapeutic agent. However, 3-Eth-5-NIdR also possesses several other important pharmacological and pharmacokinetic features that make it distinct from conventional nucleoside analogues. Figure 4D highlights three important distinctions between 3-Eth-5-NIdR and conventional nucleosides. First, 3-Eth-5-NIdR contains a unique nucleobase devoid of classical hydrogen-bonding functional groups. Second, the presence of an ethynyl moiety allows for facile covalent attachment of fluorogenic molecules. Finally, 3-Eth-5-NITP is a natural deoxyribose moiety rather than a modified sugar. All three features significantly impact the biological function of 3-Eth-5-NIdR. As discussed earlier, the presence of the non-natural nitro moiety allows for TdT, a specialized DNA polymerase involved in replicating DSBs, to efficiently incorporate S-NITP and 3-Eth-5-NITP. This feature promotes selectivity to inhibit template-independent DNA synthesis. Indeed, the increased utilization of these analogues by TdT provides a reasonable mechanism to explain their higher potencies against ALL cells with elevated TdT levels compared to those with lower levels of TdT.

While conventional nucleoside analogues function as effective therapeutic agents against leukemia, none possess diagnostic capabilities to monitor their self-effectiveness. The work provided here demonstrates proof-of-concept for the use of 3-Eth-5-NIdR as a valuable theranostic agent against leukemia. The presence of an ethynyl moiety was used to directly and selectively attach a fluorogenic probe to the non-natural nucleotide after it was incorporated into DNA. This reaction not only validates the mechanism of action of the nucleoside at the cellular level but also confirms its diagnostic potential by equating the amount of nucleotide incorporation into genomic DNA with the therapeutic activity of the corresponding non-natural nucleoside. This technology represents an important clinical feature that can be used to directly quantify the location and concentration of the therapeutic nucleotide in patient samples. Indeed, theranostic agents such as 3-Eth-5-NIdR can allow physicians to adjust the dose of nucleoside rapidly and accurately to optimize its therapeutic effectiveness. These features could alleviate possible adverse side effects associated with chemotherapy capability and thus revolutionize patient care by achieving optimal levels of a drug to kill cancer cells without harming normal cells.

The final point to emphasize is that the inclusion of a natural deoxyribose moiety may also provide improved pharmacokinetic features compared to conventional nucleoside analogues. For example, natural nucleosides and their anti-cancer counterparts enter cells via the activity of various equilibrative and concentrative nucleoside transporters. The major recognition element for efficient transport is the presence of a natural (deoxy)sugar moiety. Transport activity is highly sensitive to the correct sugar conformation as nucleoside analogues such as AZT (zidovudine) and ddC (zalcitabine) that lack a 3'-hydroxyl group show poor cellular uptake. As such, the presence of an unmodified deoxyribose group on 5-NIdR and 3-Eth-5-NIdR may facilitate their cellular transport and provide an advantage over conventional nucleoside analogues such as fludarabine and gemcitabine that use modified sugar moieties to inhibit DNA synthesis. The presence of a correct deoxyribose moiety on 5-NIdR and 3-Eth-5-NIdR may also facilitate their conversion to the triphosphate form that is required for incorporation into nucleic acid. However, one concern regarding the potential use of 5-NIdR as a therapeutic agent in humans or in animal models is the presence of the nitro pharmacophore. It is well established that certain nitroaromatic compounds can undergo nitroreductive bioactivation reactions that can generate potentially reactive nitrosoanion radicals,
nitroso intermediates, and N-hydroxy derivatives that can react with biological nucleophiles (DNA, RNA, and/or protein).\textsuperscript{50} Indeed, therapeutic agents such as flutamide and nitrofurantoin that contain nitroaromatic moieties can produce idiosyncratic liver injury. However, the presence of a nitro moiety on a drug does not unequivocally indicate a high risk for producing toxic effects. For example, several biologically active nitroheterocyclic compounds such as 2- and 5-nitrimidazoles and 5-nitrofurans function as therapeutic agents against protozoan and microbial infections.\textsuperscript{51} Regardless, these discussions make it clear that metabolic and toxicological studies are needed to evaluate the safety of 5-NIdR as a potential theranostic agent.

### METHODS

**Materials.** Magnesium acetate, MgCl₂, and Trizma base were from Sigma. [γ-32P]-ATP was purchased from Perkin-Elmer Life. Urea, acrylamide, and bis-acrylamide were from National Diagnostics. All oligonucleotides were synthesized by Operon Technologies. Antibodies directed against TdT were from Santa Cruz Biotechnology. The primary antibody for β-actin was obtained from Cell Signaling Technology, Inc. Horseradish peroxidase (HRP)-conjugated anti-mouse antibody was from Jackson Immunoresearch Laboratories, Inc. All other materials were obtained from commercial sources and were of the highest quality available. Terminal deoxynucleotidyl transferase was purchased from Fisher Scientific. MOLT4, CEM-C7, Jurkat, Loucy, J54.01, and RS4(11) cell lines were purchased from ATCC. Magnesium acetate, MgCl₂ and Trizma base were from Fisher Scientific, containing a complete protease inhibitor cocktail (Thermo Scientific), and the intensities of TdT and β-actin bands. Data represents an average of three independent determinations.

**Polymerization Assays.** All enzymatic assays were performed as previously described.\textsuperscript{52} Briefly, V₅₀, Kₘ, and V₅₀ values were determined using pseudo-first-order reaction conditions in which 6 units of TdT was preincubated with single-stranded DNA substrate (1.5 μM) in an assay buffer and mixed with variable concentrations of the nucleotide analogue (0.05–50 μM). Reactions were quenched with 200 mM EDTA at variable times (5–600 s) and analyzed using denaturing gel electrophoresis. Time courses in product formation were fitted using eq 1:

\[
Y = mt + b
\]

where \(Y\) is the amount of product, \(m\) is the rate of the reaction, \(t\) is time, and \(b\) is the \(Y\)-intercept. \(Kₘ\) and \(V₅₀\) values were determined by fits of the data points to the Michaelis–Menten equation:

\[
\text{rate} = \frac{V_{max} [dNTP]}{Kₘ + [dNTP]}
\]

where \(V_{max}\) is the maximal rate of nucleotide incorporation, \(Kₘ\) is the Michaelis constant for dNTP, and \([dNTP]\) is the concentration of nucleotide substrate.

The IC₅₀ value for 3-Eth-5-NITP was obtained using a nonlinear regression curve fit of the data to eq 3:

\[
Y = \frac{100}{1 + \frac{IC_{50}}{[inhibitor]}}
\]

where \(Y\) is the fraction of TdT activity, \(IC_{50}\) is the concentration of 3-Eth-5-NITP to inhibit 50% TdT activity, and [inhibitor] is the concentration of 3-Eth-5-NITP tested. The Cheng-Prusoff equation (eq 4) was used to define a true \(K_i\) value for 3-Eth-5-NITP by normalizing the measured IC₅₀ value for the concentration of dNTPs and their corresponding \(K_m\) values used in the experiments.

\[
K_i = \frac{IC_{50}}{1 + \frac{[dNTP]}{K_m [dNTP]}}
\]

where \(K_i\) is the true inhibition constant for 3-Eth-5-NITP, \(IC_{50}\) is the concentration of 3-Eth-5-NITP that inhibits 50% TdT activity, \([dNTP]\) is the concentration of nucleotide substrate and \(K_m\) is the Michaelis constant for dNTP.

**Cell Culture Procedures.** All cells were cultured in a humidified atmosphere of 5% CO₂ at 37 °C. MOLT4, Jurkat, J54.01, and RS4(11) cells were maintained in ATCC-formulated RPMI-1640 media supplemented with 10% fetal bovine serum (FBS), 5% l-glutamine, and 2.5% penicillin/streptomycin. Loucy and CEM-C7 cells were maintained in Cellgro formulated RPMI-1640 supplemented with 10% heat-inactivated FBS, 5% l-glutamine, and 2.5% penicillin/streptomycin antibiotic. Cells were routinely propagated and used for experiments in logarithmic phase.

**Western Blot Analysis of TdT Present in ALL Cell Lines.** Cell lysates from each ALL cell line were prepared in RIPA buffer (Thermo Fisher Scientific) containing a complete protease inhibitor cocktail tablet. The lysates (each with 50 μg of total protein content) were loaded on a Novex 4%–20% Tris-Glycine gel, transferred into 0.2 μm nitrocellulose membrane and blocked with 1X Blocking buffer (Sigma-Aldrich) overnight at 4 °C. The membrane was probed for TdT with primary antibody diluted in blocking solution (1:200) for 1 h at RT and then with a secondary donkey anti-goat IgG-HRP (1:5000 dilution) under identical conditions as the primary antibody. Immunoblots were developed by using enhanced chemiluminescence (Thermo Scientific), and the intensities of TdT and β-actin bands were quantified using the ImageJ program (http://imagej.nih.gov/ij/). TdT in each cell line was normalized by the corresponding β-actin content defined by Western blot analysis under identical conditions (TdT Content = the intensity of TdT band divided by the intensity of β-actin band). Data represents an average of three independent determinations.

**Cell Proliferation Assays.** Cells were seeded at a population density of ~200,000 cells mL⁻¹ and treated with variable concentrations of non-natural nucleoside (0.1–100 μg mL⁻¹) for up to 72 h. The final DMSO concentration in all experiments was 0.1%. Cell viability was assessed via trypan blue staining and counting the number of viable (clear) versus non-viable (blue) cells under a microscope using a hemocytometer. The IC₅₀ values for both non-natural nucleosides were obtained using a nonlinear regression curve fit of the data to eq 3. LD₅₀ values for the non-natural nucleosides were calculated using identical approaches.

**Apoptosis Measurements.** Cells were treated with DMSO (vehicle), 5-NIdR (100 μg mL⁻¹), and 3-Eth-5-NIdR (10 and 40 μg mL⁻¹) as described above. Cells were harvested by centrifugation and washed in phosphate-buffered saline and resuspended in 100 μL of binding buffer containing AnnexinV-Alexa Fluor 488 conjugate. Cells were treated with propidium iodide (PI) and incubated at RT for 15 min followed by flow cytometry analysis. This enables live cells (unstained with either Alexa Fluor 488 or PI) to be discriminated from early apoptotic (stained with Alexa Fluor 488), late apoptotic cells (stained with Alexa Fluor 488 and PI), and necrotic cells (stained with PI).

In situ "click" reactions were performed using cells harvested after 2 days of treatment with DMSO, 3-Eth-5-NIdR (10, 40, or 100 μg mL⁻¹), 3-NIdR (100 μg mL⁻¹) or EdU (20 μM). All cells were fixed with cold methanol. Under minimal lighting, cells were treated with 0.3 mL of saponin-based permeabilization and wash buffer for 45 min at 37 °C. Click reactions were initiated by adding the click-iT reaction cocktail according to the instructions of the manufacturer (Invitrogen) followed by incubation at 37 °C for 90 min. Cells were then washed twice with saponin-based permeabilization and wash buffer. Cell pellets were dislodged using 0.5 mL solution of 10 μg mL⁻¹ PI and
RNase A in saponin-based permeabilization and wash buffer and then incubated for at least 15 min prior to flow cytometry analysis.

## ASSOCIATED CONTENT

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#### Notes

The authors declare no competing financial interest.

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