Synthesis of N^3-Substituted Thymidine Analogues for Measurement of Cellular Kinase Activity

P. Ghosh[#], A. Pal, A. Shavrin, W. Bornmann, J.G. Gelovani and M.M. Alauddin[*]

Department of Experimental Diagnostic Imaging, The University of Texas M. D. Anderson Cancer Center, Houston, TX, USA

Abstract: N^3-Substitued thymidine analogues that carry a carboranylalkyl moiety at the N^3-position with various spacer lengths have been reported to be good substrates for thymidine kinase (TK1). As part of our continuing effort towards the development of new TK1 substrates for imaging tumor proliferative activity, we have synthesized a series of new N^3-substituted analogues of thymidine that carry an aromatic ring with different spacer lengths. The overall yields for **6** and **7** were 13% and 39% in four steps and three steps, respectively, and those for **14**, **16** and **18** were in the range of 13%-15% in six steps. The overall yield for **24** was 33% in three steps, and those for **25** and **26** were 64% and 58%, respectively, in one step. Most of these compounds have been tested for TK1 activity by enzymatic assay to identify a good substrate that can be radiolabeled for imaging. The phosphorylation rates of these compounds were 2%-6% compared with that of thymidine. The results from the *in vitro* enzymatic assays suggest that these N^3-substituted thymidine analogues have some potential for imaging TK1 activity if radiolabeled with a suitable isotope.

Key Words: Nucleoside analogues, N^3-substituted thymidine, thymidine kinase, TK1, ATP.

INTRODUCTION

Uncontrolled cell division is the defining characteristic of cancer, therefore, many anticancer therapies are aimed directly at inhibiting cell division in tumors [1]. The metabolic process most directly related to cell division is DNA synthesis, while one of the building blocks of DNA is thymidine [2]. Cells form thymidylate (thymidine monophosphate), a necessary precursor of DNA, either by methylation of deoxyuridine monophosphate ("*de novo* pathway") or phosphorylation of thymidine (TdR) imported from the extracellular milieu ("salvage pathway") [1, 3]. In the salvage pathway, thy-midine gets phosphorylated by the cellular kinases such as thymidine kinases TK1 and TK2. TK1 is up regulated in the S-phase of the cell cycle [4]. Thymidine labeled with ^{3}H or ^{14}C has been used for decades to measure cell proliferation [5]. Investigators in the field of nuclear medicine have been seeking to develop radiotracers for non-invasive imaging of cell proliferation for more than 30 years [6]. Thymidine has been radiolabeled with ^{11}C ($t_{1/2}$=20 min) for imaging cell proliferation and tumor activity by positron emission tomography (PET) [6-8]. However, the *in vivo* catabolism of thymidine limits its application for this purpose [3, 9]. Many fluorinated analogues of thymidine have been developed as antiviral agents through modification of the sugar and the base [10, 11]. These include 2′-deoxy-2′-fluoro-5-methyl-1-β-D-arabinofuranosyluracil (FMAU), 2′-deoxy-2′-fluoro-5-iodo-1-β-D-arabinofuranosyluracil (FIAU) and other 5-substituted analogues, and 3′-deoxy-3′-fluorothymidine (FLT) [10-12]. These analogues are more resistant to catabolic degradation *in vivo* than thymidine, and some of them have been radiolabeled with suitable isotopes for PET imaging of cellular proliferation and herpes simplex virus thymidine kinase (HSV-tk) gene expression [12-15].

We have been developing and testing radiolabeled pyrimidine nucleoside analogues as potential agents for imaging tumor proliferative activity and HSV-tk reporter gene expression [13, 15-21]. Similarly, others have reported several radiolabeled nucleoside analogues for these purposes [12, 22]. Among these thymidine analogues, FLT, FMAU, and other 5-substituted derivatives are known to be phosphorylated by human and other mammalian nucleoside kinases including TK1 and TK2; viral kinases such as herpes simplex virus (HSV) type 1 and 2; and hepatitis B-virus [10, 11, 14, 18-24]. In particular, ^{11}C-FMAU and ^{18}F-FMAU are being investigated in clinical studies at multiple centers for imaging tumor proliferation in a variety of cancer types [25] and for imaging DNA synthesis [26, 27]. 2′-Deoxy-2′-fluoro-5-methyl-1-β-D-ribofuranosyluracil (FMRU) and 5-substituted analogues have also been synthesized and tested as potential imaging agents for tumor proliferation [28, 29].

To date, two nucleoside analogues have been radiolabeled in the 3′-position: ^{18}F-FLT [12] and 3′-deoxy-3′-^{18}F-fluoro-5-methyl-1-β-D-xylo-furanosyluracil (^{18}F-FMXU) [17]. ^{18}F-FLT is currently under clinical investigation as a PET imaging agent for tumor proliferation [30, 31]. However, the phosphorylation rates of FLT and FMAU by TK1 are relatively low (10%-15%) compared with that of thymidine [32]. Therefore, it is necessary to identify other nucleoside analogues that can be phosphorylated by TK1 with high phosphorylation rates, that are resistant to degradation by thymidine phosphorylase and that can be radiolabeled with a suitable isotope for imaging.

*Address correspondence to this author at the Department of Experimental Diagnostic Imaging, T8.3895, Box 059, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030, USA; Tel: 713-563-4872; Fax: 713-563-4894;
E-mail: alauddin@di.mdacc.tmc.edu

#Current Address: Department of Radiology, The Methodist Hospital Research Institute, Houston, TX 77030, USA

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During the past decade, a series of N^3-substituted-thymidine analogues that carry a carboranylalkyl moiety at the N^3-position, with spacers of various lengths, have been developed for boron neutron capture therapy [33-37]. Some of these N^3-substituted-thymidine analogues have been shown to be phosphorylated by TK1 with 50%-89% efficacy compared with thymidine, but are poor substrates for thymidine phosphorylase [35-37]. Several N^3-substituted thymidine derivatives with straight chains, such as ethyl, n-butyl and acetylene, have also been reported to be effective substrates for TK1, with relatively high phosphorylation rates [31, 36, 38]. These favorable properties of N^3-substituted thymidine analogues prompted us and others to develop ^{18}F-labeled N^3-substituted-thymidine analogues for use in PET imaging of tumor proliferative activity (*via* TK1 activity) [39, 40].

We hypothesized that a bulky lipophilic group, such as a phenyl group, at the end of the tether would mimic the carboranyl group of previously reported N^3-substituted compounds [35-37], which have been reported to be phosphorylated by TK1 with 50%-89% efficiency relative to thymidine and are resistant to degradation by thymidine phosphorylase. We recently reported on the synthesis and radiosynthesis of two new N^3-substituted thymidine analogues with chains of different lengths, each of which contains an aromatic substituent at the end of the spacer [41]. In the current paper, we report on the synthesis of an additional series of N^3-substitued thymidine analogues and their substrate affinity to TK1 enzyme.

RESULTS AND DISCUSSION

Chemistry

The synthetic scheme for **6** and **7** is shown in Fig. (**1**). 3′,5′-bis-Tetrahydropyranylthymidine **(1)** was prepared from thymidine in 95% yield using a literature method [42].

1,2-Dibromo-4-phenyl butane **(2)** was obtained in 82% yield. Thymidine THP ether (**1**) was reacted with **2** following a literature method [37] to produce **3** and **4**. The reaction mixture was kept at room temperature for 8 h and then refluxed at 50°C for 12 h. At room temperature, **3** was produced in good yield, however, at 50°C, the reaction eventually produced **4** as a by-product with a moderate yield. The mixture of **3** and **4** was separated by purification on a silica gel column using 25% acetone in hexane. The yields in this step were 56% and 33% for **3** and **4**, respectively. Compound **4** is likely to be a hydrolyzed product of **3** due to the presence of a trace of water in the reaction mixture.

3′,5′-O-bis-Tetrahydropyranyl-N^3-(2-chloro-4-phenylbutyl)thymidine (**5**) was obtained from a reaction of **4** with methane sulfonyl chloride (MsCl) in dichloromethane (CH_2Cl_2) in the presence of triethylamine and N,N-dimethyl aminopyridine (DMAP). The reaction mixture was purified on a silica gel column and isolated in 69% yield. Our intention was to prepare a mesylate derivative that could be fluorinated to the respective fluoro compound. Interestingly, the mesylate was too reactive to isolate; instead, it was converted to the chloroderivative, and a small amount of mesy-

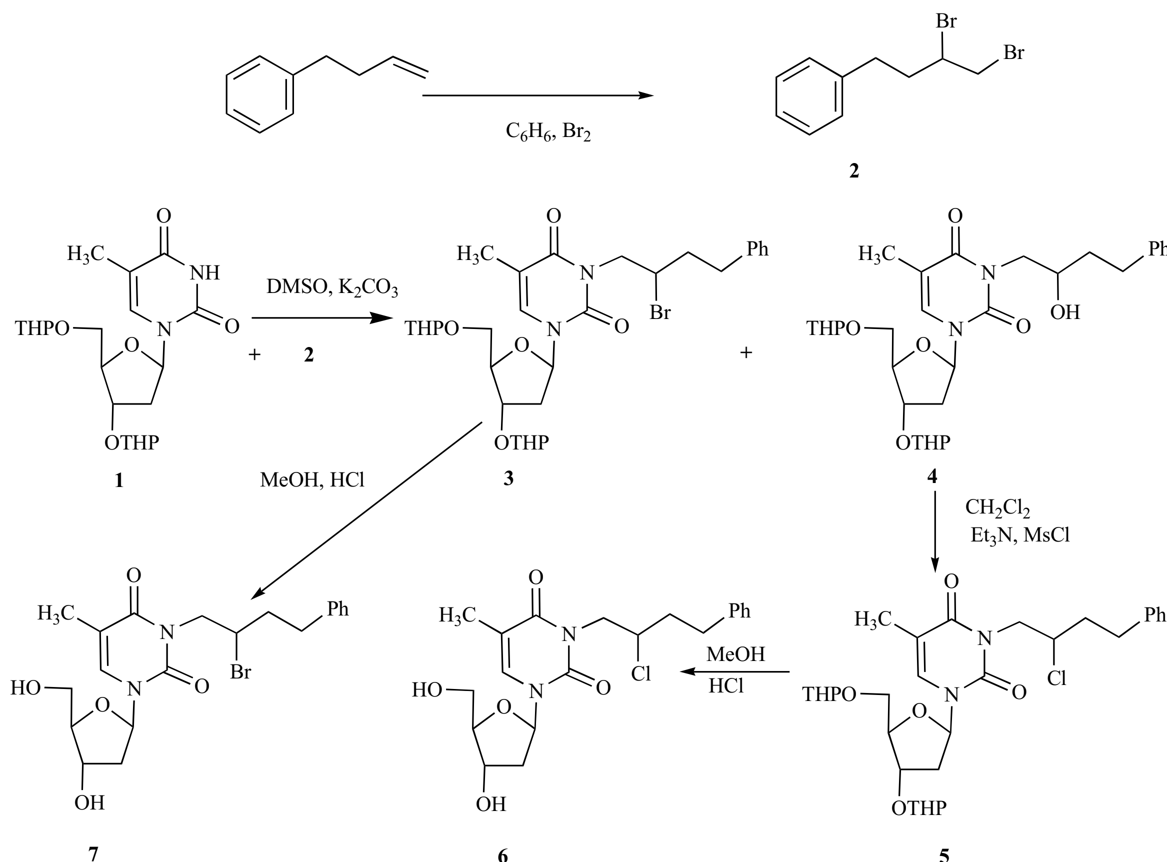

Fig. (1). Scheme 1; for synthesis of compounds **6** and **7**.

late could be isolated from some reactions. Acid hydrolysis of **5** and **3**, followed by flash chromatography, produced **6** and **7** in 77% and 86% yields, respectively. The overall yield of **6** was 13% in four steps and that of **7** was 39% in three steps. These compounds were fully characterized by ^{1}H nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry.

Fig. (**2**) represents the synthesis of N^3-substituted thymidine analogues **14**, **16** and **18**. 1-Phenyl-1,5-pentanediol (**8**) was obtained from a commercial source and reacted with *p*-toluenesulfonyl chloride (TsCl) in CH_2Cl_2 in the presence of triethylamine and DMAP to produce **9** in 80% yield after purification. 5-*p*-Toluenesulfonyl-1-phenylpentylbenzoate (**10**) was prepared by treatment of **9** with benzoic anhydride in CH_2Cl_2 in the presence of triethylamine and DMAP. The yield in this step was 71% after purification by flash chromatography. Reaction of **10** with **1** in acetone and DMSO (1:1) and potassium carbonate at 50°C for 22 h produced **11** in 66% yield. Hydrolysis of **11** with sodium hydroxide in MeOH followed by purification on a silica gel column produced **12** in 84% yield.

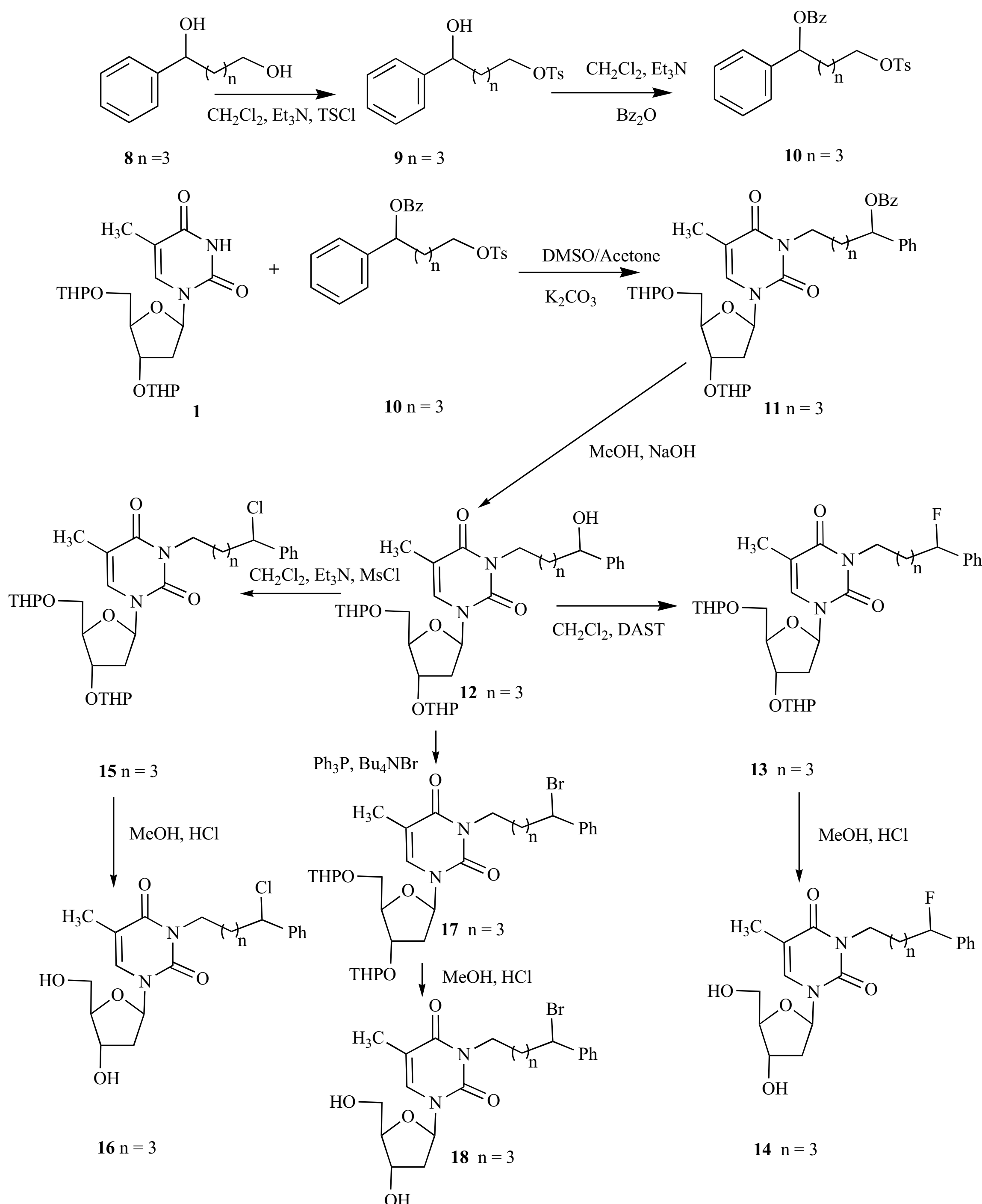

Fig. (2). Scheme 2; for synthesis of compounds **14**, **16** and **18**.

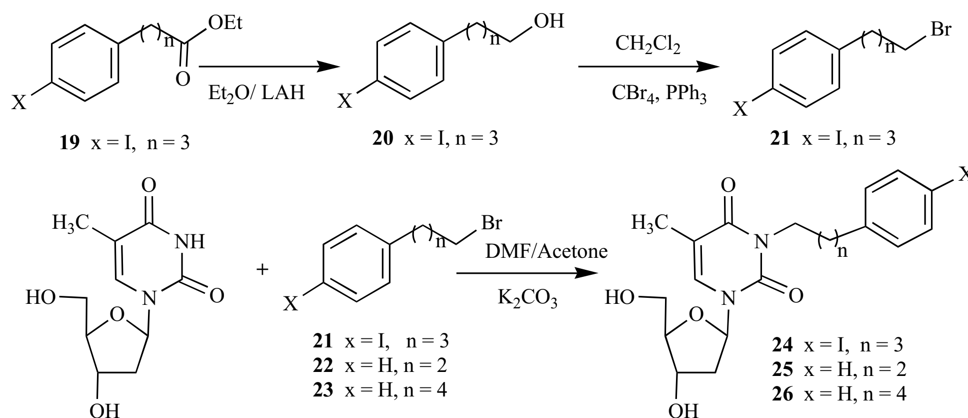

Fig. (3). Scheme 3; for synthesis of compounds **24**, **25** and **26**.

Reaction of **12** with diethylaminosulfur trifluoride (DAST) in CH_2Cl_2 at -78°C produced **13** in 54% yield. The compound was characterized by ^{19}F-NMR spectroscopy in addition to the proton NMR. Hydrolysis of **13** by acid in methanol produced **14** in 75% yield. Reaction of **12** with MsCl in CH_2Cl_2 in the presence of triethylamine and DMAP at 50°C produced **15**. The crude product was purified on a silica gel column and isolated in 60% yield. In this reaction our attempt was to prepare mesylate derivative for potential fluorination and radiofluorination; however, we were unable to isolate a mesylate for **12.** Instead, the isolated product was a chloro-derivative **15**, possibly due to the instability of the mesylate under the reaction condition. A bromo-derivative **17** was prepared from **12** by reaction with tetrabutylammonium bromide in the presence of triphenylphosphine and 2,3-dichloro-5,6-dicyanobenzoquinone at room temperature [43]. The yield in this step was 58%. The compound was fully characterized by spectroscopic methods. Acid hydrolysis of **15** and **17** produced **16** and **18** in 83% and 80% yields, respectively. The overall yields of **14**, **16** and **18** were 13%-15% in six steps. All these compounds were characterized by ^{1}H NMR spectroscopy and high resolution mass spectrometry. The ^{1}H NMR spectrum of compound **14** revealed a peak (2 m) centered at 5.45 ppm with J=48.0 Hz, a typical geminal coupling constant between fluorine and hydrogen. ^{19}F NMR spectrum (coupled) showed a multiplet centered at -174.20 ppm. The methyl (CH_3) protons appeared as a doublet with small coupling constant of 0.9 Hz.

The synthetic scheme for **24**, **25** and **26** is shown in Fig. (**3**). Compounds **19**, **22** and **23** were obtained from commercial sources, and **19** was reduced with lithium aluminium hydride to prepare **20** in 80% yield. The aromatic alcohol **20** was then reacted with triphenyl phosphine and carbon tetrabromide in CH_2Cl_2 to produce **21** in 68% yield. Thymidine was coupled separately with **21**, **22** and **23** following a literature method [44]. Thus, the reaction of one equivalent of thymidine with two equivalents of these bromoalkanes in dry acetone:DMF (1:1) under basic conditions (K_2CO_3) yielded the N^3-substituted derivatives of thymidine **24**, **25** and **26** in 61%, 64% and 58% yields, respectively.

Enzymatic Assay

An *In vitro* enzymatic assay of these compounds was performed following a method reported in the literature [34]. Thymidine was used as the positive control, which was phosphorylated by TK1 enzyme when treated with ^{32}P-labeled adenosine triphosphate (^{32}P-ATP) in the presence of the TK1 enzyme. All compounds including thymidine were treated with TK1 and ^{32}P-ATP under identical reaction conditions. Thymidine was phosphorylated by TK1, resulting in high yields. The amount of thymidine monophosphate formed by this enzymatic transfer of phosphate was considered to be 100%. We attempted to analyze the crude phosphorylated products using thin layer chromatography (TLC) as described in the literature [34]. However, separation between the nucleoside mono-phosphate and ATP was poor with TLC for these compounds compared with the reported carboranyl derivatives of the N^3-substituted thymidine [34]. Therefore, to analyze the phosphorylated compounds, we used high-performance liquid chromatography (HPLC) according to a reported method; HPLC can separate the nucleoside and nucleotides very well without any ambiguity [18].

Fig. (**4**) represents HPLC chromatograms of a standard mixture of thymidine phosphates and radio-chromatograms generated by plotting the radioactivity in each collected fraction from HPLC with time. Fig. (**4A**) is the cold standard mixture of thymidine (TdR), thymidine monophosphate (TdRMP), thymidine diphosphate (TdRDP) and thymidine triphosphate (TdRTP), which were well resolved under HPLC conditions. Fig. (**4B**) shows the radiochromatogram from the HPLC analysis of a crude reaction mixture of thymidine treated with TK1 and ^{32}P-ATP. As the figure shows, radiolabeled TdRMP eluted at 6.0 min (a), and unlabeled TdRMP eluted at 5.75 min. The small difference in retention time between the unlabeled TdRMP and the radioactive peak (TdRMP) was due to the delay time between the UV detector and fraction collection. The ^{32}P-phosphate transferred from ATP was quite high for thymidine monophosphorylation. In addition to thymidine monophosphate, there was an unknown radioactive peak (b) that was observed in most of the reactions between ATP and nucleosides in the presence of TK1.

Fig. (**4C**) shows a representative radiochromatogram of N^3-substituted thymidine analogues (compound **16**). The 6.0-min fraction had minimal radioactivity, similar to that of thymidine monophosphate; this activity (nucleoside mono-

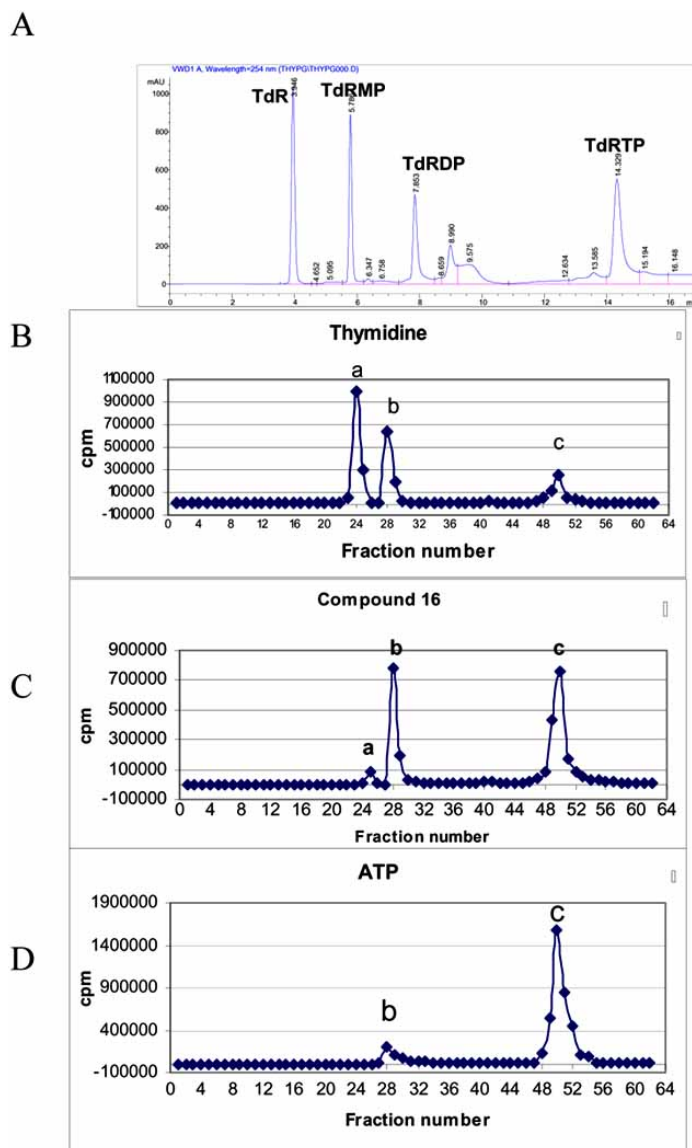

Fig. (4). HPLC chromatograms: Ion exchange column (Partisil 10 SAX, Whatman, NY). Flow rate 1 mL/min, with a linear gradient from 100% A (10 mM potassium dihydrogen phosphate in water, pH 3.7) and 0% B (1 M potassium dihydrogen phosphate in water, pH 3.7) to 0% A and 100% B over a 16-min period.

4A: UV trace of a mixture of thymidine phosphates (TdR, TdRMP, TdRDP, and TdRTP).

4B: Radiochromatogram of the reaction mixture of thymidine with ^{32}P-ATP and TK1. **a:** TdRMP; **b:** unknown; **c:** ATP.

4C: Radiochromatogram of the reaction mixture of N^3-substituted thymidine **16** with ^{32}P-ATP and TK1. **a:** Nucleoside-MP; **b:** unknown; **c:** ATP.

4D: Radiochromatogram of standard ^{32}P-ATP. **b:** unknown; **c:** ATP.

phosphate) was approximately 6% compared with that of thymidine. An analysis of standard ^{32}P-ATP showed an unknown peak at approximately 7 min (Fig. **4D**), which was approximately 10% of the total activity. Nucleoside triphosphates are known to be labile, especially at high temperatures, and lose phosphate to convert into diphosphates. The unknown peak at 7 min in the standard ^{32}P-ATP is likely to be free radioactive phosphate due to decomposition, and this decomposition was enhanced when the reaction mixtures were heated up to 90°C to stop enzymatic activity. This unknown peak eluted at 7 min was well separated from the monophosphate by HPLC, but it was not separated by TLC; therefore, it could be mistaken for nucleoside monophosphate when analyzed by TLC. Other compounds tested for enzymatic assay showed similar results: i.e., approximately 2%-6% phosphorylation by TK1 (Table **1**). These results are in agreements with those reported recently for N^3-substituted thymidine analogues labeled with Tc99m [45]. An HPLC analysis of the crude reaction mixture provided more accurate measurement and identification of the early eluting peaks; therefore, this method appears to be more reliable than TLC.

Table 1. Phosphorylation of N^3-Substituted Thymidine Analogues Compared with Thymidine

Compounds	% Phosphorylation
Thymidine	100
6	N/A
7	3
14	2
16	6
18	N/A
24	4
25	3
26	2

We have been developing PET imaging agents to measure TK1 activity in proliferating cells. We have now synthesized several new N^3-substituted thymidine analogues that carry an aromatic ring with various spacer lengths, resulting in good yields. Preliminary enzymatic assays have been performed on most of these compounds. The results from these *in vitro* enzymatic assays suggest that these N^3-substituted thymidine analogues are less efficient substrates for TK1 compared with thymidine. For comparison, the relative accumulation rate of FLT, another thymidine analogue currently used for PET imaging of proliferative activity, is 10%-13% compared with thymidine. Thus, the described N3-substitued compounds may have potential for imaging tumor proliferative activity, because they are only 2-6 folds less efficient than FLT as substrates for TK1. Since FLT does not cross the intact blood-brain barrier (BBB) due to polar nature and lack of thymidine transporters on the luminal side, the more lipophilic N^3-substituted analogues may have an advantage. Furthermore, the N^3-substituted thymidine analogues have been reported to be resistant to degradation by thymidine phosphorylase, which may provide an additional advantage in terms of low levels or no radiolabeled catabolites circulating in the blood, which contribute negatively to background activity in normal and tumor tissues and complicate the interpretation of imaging results. The pharmacokinetic modeling and parametric imaging of tumor proliferative activity may be simplified with N^3-substituted thymidine analogues. However, further studies, including *in vitro* cell uptake and *in vivo* biodistribution and tumor uptake will be necessary to confirm the efficacy of these compounds against TK1 activity.

Conclusion

Syntheses of several new N^3-substituted thymidine analogues that carry an aromatic ring with various spacer lengths have been achieved in good yields. The results from the *in vitro* enzymatic assays suggest that these N^3-substituted thymidine analogues have some potential for imaging TK1 activity if radiolabeled with a suitable isotope.

EXPERIMENTAL SECTION

Reagents and Instrumentation

All reagents and solvents were purchased from Aldrich Chemical Co. (Milwaukee, WI), and used without further purification unless otherwise specified. TLC was performed on pre-coated Kieselgel 60 F254 (Merck, Darmstadt, Germany) glass plates. Proton and ^{19}F NMR spectra were recorded on a Brucker 300 MHz spectrometer using tetramethylsilane as an internal reference and hexafluorobenzene as an external reference, respectively, at The University of Texas M. D. Anderson Cancer Center. Low-resolution mass spectral analysis was performed in-house with an HPLC mass spectrometer (Applied Biosystems Q-Trap LC/MS/MS). High-resolution mass spectra were obtained on a Brucker BioTOF II mass spectrometer at the University of Minnesota using electrospray ionization (ESI) technique.

HPLC was performed on an 1100 series pump (Agilent, Germany), with a built-in UV detector operated at 254 nm and a radioactivity detector with a single-channel analyzer (Bioscan, Washington, DC) using a Partisil 10 SAX column (Whatman, NY). Elution was performed at a flow rate of 1 mL/min, with a linear gradient from 100% A (10 mM potassium dihydrogen phosphate in water, pH 3.7) and 0% B (1 M potassium dihydrogen phosphate in water, pH 3.7) to 0% A and 100% B over a 16-min period. Fractions were collected at 15-sec intervals up to 16 min. Radioactivity in each fraction was measured in a liquid scintillation counter and plotted with time of elution.

Preparation of 3´,5´-O-bis-tetrahydropyranylthymidine: 1

3´,5´-O-bis-Tetrahydropyranylthymidine (**1**) was prepared according to a published method [42].

Preparation of 1,2-dibromo-4-phenylbutane: 2

To a solution of 4-phenyl-1-butene (1.0 g, 7.56 mmol) in benzene (10 mL), perflurohexane (1.52 mL, 7.56 mmol) was added at 0°C over 10 min. Bromine (0.8 mL, 15.12 mmol) was added to the reaction mixture and stirred at 0°C for 1 h. The reaction mixture was warmed to room temperature and further stirred for an additional 1 h, when TLC showed that no starting material remained. The solvent was evaporated, and the crude product was purified on a silica gel column and eluted with 5% acetone in hexane. The appropriate fractions were combined and evaporated to produce 1.82 g of **2** in 82% yield. ^1H NMR **2** ($CDCl_3$) δ: 7.37 (d, 2H, J=6.0 Hz, aromatic), 7.30-7.20 (m, 3H, aromatic), 4.21-4.09 (m, 1H, C_2H), 3.91 (dd, 1H, J=4.5 Hz, J=10.5 Hz, C_1H), 3.68 (t, 1H, J=9.9 Hz, C_1H), 3.05-2.93 (m, 1H, C_4H), 2.88-2.73 (m, 1H, C_4H), 2.59-2.44 (m, 1H, C_3H), 2.19-2.05 (m, 1H, C_3H). MS: M+1, calculated 293.01; found 293.14.

Preparation of 3´,5´-O-bis-tetrahydropyranyl-N^3-(2-bromo-4-phenylbutyl)thymidine: 3

1,2-Dibromo-4-phenyl butane (**2**) (1.0 g, 3.42 mmol) was dissolved in acetone (10 mL) and DMSO (10 mL) (1:1) under argon. Potassium carbonate (1.7 g, 17.1 mmol) was added, followed by the addition of thymidine THP ether (**1**) (1.4 g, 3.42 mmol). The reaction mixture was stirred at

ambient temperature for 8 h and then heated at 50°C for 12 h, when TLC showed that no significant starting material remained. The reaction mixture was filtered and evaporated under vacuum. The residue was re-dissolved in CH_2Cl_2 (150 mL) and washed with H_2O (3x150 mL). The organic layer was dried over $MgSO_4$, filtered, concentrated under vacuum, and purified on a silica gel column using 25% acetone in hexane. The pure product **3** (1.19 g) was obtained in 56% yield. ^{1}H NMR **3** ($CDCl_3$) δ: 7.72-7.51 (m, 1H, C_6H), 7.35-7.25 (m, 2H, aromatic), 7.22-7.13 (m, 3H, aromatic), 6.42-6.32 (m, 1H, 1′H), 4.78-3.34 (m, 13H, N^3-$C_{1,2}$H, THP & 3′-5′H), 2.78-2.35 (m, 2H, C_4H), 2.25-2.00 (m, 2H, 2′H) 1.96, 1.93 (2s, 3H, CH_3), 1.88-1.47 (m, 14H, N^3-C_3H & THP). MS: M+1, calculated 622.56; found 622.43.

Preparation of 3′,5′-O-bis-tetrahydropyranyl-N³-(2-hydroxy-4-phenylbutyl)thymidine: 4

This compound was isolated from the above reaction mixture as a by-product, and 0.63 g of **4** was obtained in 33% yield. ^{1}H NMR **4** ($CDCl_3$) δ: 7.76-7.45 (m, 1H, C_6H), 7.32-7.11 (m, 5H, aromatic), 6.43-6.29 (m, 1H, 1′H), 4.78-3.47 (m, 13H, N^3-$C_{1,2}$H, THP & 3′-5′H), 2.94-2.37 (m, 2H, C_4H), 2.34-2.00 (m, 2H, 2′H), 1.97, 1.94 (2s, 3H, CH_3), 1.91-1.48 (m, 14H, N^3-C_3H & THP). MS: M+1, calculated 559.66; found 559.75.

Preparation of 3′,5′-O-bis-tetrahydropyranyl-N³-(2-chloro-4-phenylbutyl)thymidine: 5

3′,5′-O-bis-Tetrahydropyranyl-N^3-(2-hydroxy-4-phenylbutyl)thymidine (**4**) (0.5 g, 0.90 mmol) was dissolved in CH_2Cl_2 (10 mL) under argon, triethylamine (1.3 mL, 9.0 mmol) was added, followed by the addition of DMAP (33 mg, 0.27 mmol). The reaction mixture was stirred at room temperature, MsCl (140 µL, 1.8 mmol) was added and the reaction mixture was heated at 60°C for 12 h, when TLC showed that no starting material remained. The solvent was evaporated under vacuum, the residue was dissolved in CH_2Cl_2 (100 mL) and washed with H_2O (3x100 mL). The organic phase was dried ($MgSO_4$), evaporated to dryness and purified on a silica gel column. Appropriate fractions were combined and evaporated to produce 356 mg of **5** in 69% yield. ^{1}H NMR **5** ($CDCl_3$) δ: 7.70-7.48 (m, 1H, C_6H), 7.38-7.23 (m, 2H, aromatic), 7.21-7.14 (m, 3H, aromatic), 6.45-6.34 (m, 1H, 1′H), 4.77-3.33 (m, 13H, N^3-$C_{1,2}$H, THP & 3′-5′H), 2.76-2.35 (m, 2H, C_4H), 2.26-2.01 (m, 2H, 2′H) 1.96, 1.94 (2s, 3H, CH_3), 1.91-1.48 (m, 14H, N^3-C_3H & THP). MS: M+1, calculated 578.11; found 578.27.

Preparation of N³-(2-chloro-4-phenylbutyl) thymidine: 6

To a solution of **5** (0.2 g, 0.35 mmol) in MeOH (2 mL) was added hydrochloric acid (1M, 0.1 mL) at room temperature. The resulting mixture was refluxed for 20 min at 80°C, when TLC showed that no starting material remained. The reaction mixture was cooled and the solvent evaporated. The crude product was purified by flash chromatography to obtain **6** (165 mg) as a gummy material in 77% yield. ^{1}H NMR **6** ($CDCl_3$) δ: 7.36 (d, 1H, J=7.2 Hz, C_6H), 7.33-7.26 (m, 2H, aromatic), 7.20-7.15 (m, 3H, aromatic), 6.23-6.18 (m, 1H, 1′H), 4.64-4.59 (m, 2H, 3′H & C_2H), 4.02-3.80 (m, 5H, N^3-C_1H, 4′& 5′H), 2.65-2.50 (m, 2H, C_4H), 2.48-2.30 (m, 2H, 2′H), 1.95 (s, 3H, CH_3), 1.75-1.69 (m, 2H, N^3-C_3H). High-resolution MS: M+Na, calculated 431.8685; found 431.8697.

Preparation of N³-(2-bromo-4-phenylbutyl)thymidine: 7

The method of preparation of this compound was the same as described above. 3′,5′-O-bis-Tetrahydropyranyl-N^3-(2-bromo-4-phenylbutyl)thymidine (**3**) was hydrolyzed with hydrochloric acid (1M, 0.1 mL), and the crude product was purified by flash chromatography to obtain **7** as foam in 86% yield. ^{1}H NMR **7** ($CDCl_3$) δ: 7.35-7.24 (m, 3H, C_6H & aromatic), 7.21-7.13 (m, 3H, aromatic), 6.21-6.14 (m, 1H, 1′H), 4.67-4.55 (m, 2H, 3′H & C_2H), 4.00-3.78 (m, 5H, N^3-C_1H, 4′ & 5′H), 2.70-2.45 (m, 2H, C_4H), 2.43-2.27 (m, 2H, 2′H), 1.94 (s, 3H, CH_3), 1.78-1.66 (m, 2H, N^3-C_3H). High-resolution MS: M+Na, calculated 476.3195; found 476.3178.

Preparation of 5-p-toluenesulfonyl-1-phenyl pentanol: 9

1-Phenyl-1,5-pentanediol (**8**) (0.200 g, 1.11 mmol) was dissolved in CH_2Cl_2 (7.0 mL) in a dry flask under argon. Triethylamine (0.8 mL, 5.55 mmol) was added, followed by the addition of DMAP (41 mg, 0.33 mmol). The reaction mixture was cooled to 0°C, and *p*-toluenesulfonyl chloride (TsCl, 233 mg, 1.22 mmol) was added. The reaction mixture was warmed to room temperature and stirred for 2 h, at which point TLC showed that no starting material remained. The solvent was evaporated under vacuum, the residue was re-dissolved in CH_2Cl_2 (30 mL), and the solution was washed with H_2O (3x30 mL). The organic phase was dried in $MgSO_4$, filtered and the solvent was evaporated to dryness. The crude product was purified on a silica gel column and eluted with 25% acetone in hexane. The appropriate fractions were combined and evaporated to produce 298 mg of the desired product **9** in 80% yield. ^{1}H NMR **9** ($CDCl_3$) δ: 7.78 (d, 2H, J=8.1 Hz, aromatic), 7.38-7.28 (m, 7H, aromatic), 4.65-4.61 (m, 1H, C_5H), 4.01 (t, 2H, J=6.3 Hz, C_1H), 2.46 (s, 3H, CH_3), 1.83-1.59 (m, 4H, $C_{2,4}$H), 1.56-1.26 (m, 2H, C_3H). MS: M+1, calculated 335.43; found 335.51.

Preparation of 5-p-toluenesulfonyl-1-phenylpentylbenzoate: 10

5-*p*-Toluenesulfonyl-1-phenyl pentanol (**9**) (275 mg, 0.82 mmol) was dissolved in CH_2Cl_2 (8 ml) under argon, triethylamine (0.6 ml, 4.1 mmol) was added, followed by the addition of DMAP (30 mg, 0.25 mmol). Benzoic anhydride (223 mg, 0.99 mmol) was added to the reaction mixture and stirred at room temperature for 3 h, when TLC showed that no starting material remained. The solvent was evaporated under vacuum, the residue was re-dissolved in CH_2Cl_2 (30 mL) and washed with H_2O (3x30 mL). The organic layer was dried over $MgSO_4$, filtered, concentrated under vacuum, and purified by flash column chromatography using 25% acetone in hexane as the eluent. The pure product **10** (255 mg) was obtained in 71% yield. ^{1}H NMR **10** ($CDCl_3$) δ: 8.07 (d, 2H, J=8.4 Hz, aromatic), 7.76 (d, 2H, J=8.4 Hz, aromatic), 7.56 (t, 1H, J=7.2 Hz, aromatic), 7.46 (t, 2H, J=7.8 Hz, aromatic), 7.41-7.27 (m, 7H, aromatic), 5.94 (t, 1H, J=7.2 Hz, C_5H), 4.02 (t, 2H, J=6.3 Hz, C_1H), 2.43 (s, 3H, CH_3), 2.11-1.80 (m, 2H, C_4H), 1.78-1.62 (m, 2H, C_2H), 1.55-

1.32 (m, 2H, C_3H). MS: M+1, calculated 439.54; found 439.40.

Preparation of 3′,5′-O-bis-tetrahydropyranyl-N^3-(5-phenyl-5-benzoyl pentyl)thymidine:11

A solution of **10** (260 mg, 0.59 mmol) in acetone (5 mL) and DMSO (5 mL) (1:1) under argon was added to a dry flask containing potassium carbonate (292 mg, 1.95 mmol) and **1** (243 mg, 0.59 mmol). The reaction mixture was heated with stirring at 50°C for 22 h, when TLC showed that no significant starting material remained. The reaction mixture was filtered and concentrated under vacuum and the residue was dissolved in CH_2Cl_2 (50 mL). The solution was washed with H_2O (3x50 mL). The organic layer was dried over $MgSO_4$, filtered, concentrated under vacuum and purified by flash chromatography on a silica gel column using 25% acetone in hexane as the eluent. The pure compound **11 (**264 mg) was obtained in 66% yield. ^{1}H NMR **11** ($CDCl_3$) δ: 8.08 (d, 2H, J=7.2 Hz, aromatic), 7.59-7.29 (m, 9H, C_6H & aromatic), 6.42-6.34 (m, 1H, 1′H), 5.97 (t, 1H, J=6.0 Hz, N^3-C_5H), 4.76-3.48 (m, 12H, N^3-C_1H, THP & 3′-5′H), 2.58-2.32 (m, 2H, 2′H), 2.17-1.96 (m, 2H, N^3-C_4H), 1.93, 1.89 (2s, 3H, CH_3), 1.87-1.45 (m, 16H, N^3-$C_{2,3}H$ & THP). MS: M+1, calculated 677.80; found 677.91.

Preparation of 3′,5′-O-bis-tetrahydropyranyl-N^3-(5-phenyl-5-hydroxypentyl)thymidine: 12

To a solution of **11** (280 mg, 0.41 mmol) in MeOH (3 mL) was added an aqueous sodium hydroxide solution (1M, 0.2 mL) at room temperature. The resulting solution was refluxed for 30 min at 80°C, at which point TLC showed that no starting material remained. The reaction mixture was cooled and the solvent evaporated. The crude product was purified on a silica gel column and eluted with 30% acetone in hexane to produce **12** (198 mg) in 84% yield. ^{1}H NMR **12** ($CDCl_3$) δ: 7.64, 7.58, 7.54, 7.52 (4d, 1H, J=1.2 Hz, C_6H), 7.37-7.50 (m, 5H, aromatic), 6.43-6.32 (m, 1H, 1′H), 4.78-3.49 (m, 13H, N^3-$C_{1,5}H$, THP & 3′-5′H), 2.58-2.33 (m, 2H, 2′H), 2.25-1.98 (m, 2H, N^3-C_4H), 1.94, 1.91, (2s, 3H, CH_3), 1.89-1.45 (m, 16H, N^3-$C_{2,3}H$ & THP). MS: M+1, calculated 573.69; found 573.84.

Preparation of 3′,5′-O-bis-tetrahydropyranyl-N^3-(5-phenyl-5-fluoropentyl)thymidine: 13

Diethy1aminosulfur trifluoride (DAST, 36 μL, 0.27 mmol) was dissolved in CH_2Cl_2 (3 mL) in a dry flask under argon and stirred at -78°C for 10 min. A solution of **12** (120 mg, 0.21 mmol) in dry CH_2Cl_2 (1 mL) was added dropwise *via* a syringe over a period of 3 min. The reaction mixture was stirred at -78°C for 30 min, when TLC showed that no starting material remained. The solvent was evaporated under vacuum, the residue was dissolved in CH_2Cl_2 (15 mL), and the solution was washed with H_2O (3x15 mL). The organic layer was dried over $MgSO_4$, filtered, concentrated under vacuum, and purified by flash chromatography on a silica gel column. Appropriate fractions were combined and evaporated to produce 65 mg of **13** in 54% yield. ^{1}H NMR **13** ($CDCl_3$) δ: 7.64, 7.58, 7.55, 7.53 (4d, 1H, J=1.2 Hz, C_6H), 7.43-7.30 (m, 5H, aromatic), 6.43-6.34 (m, 1H, 1′H), 5.45 (2m, 1H, J_{HF}=47.7 Hz, N^3-C_5H), 4.78-3.48 (m, 12H, N^3-C_1H, THP & 3′-5′H), 2.59-2.35 (m, 2H, 2′H), 2.33-1.98 (m, 2H, N^3-C_4H), 1.96, 1.92 (2s, 3H, CH_3), 1.89-1.42 (m, 16H, N^3-$C_{2,3}H$ &THP). ^{19}F NMR **13** (δ): -174.94 (s, decoupled), -174.66 to -175.14 (m, coupled). MS: M+1, calculated 575.68, found 575.75.

Preparation of (5-phenyl-5-fluoropentyl)thymidine: 14

To a solution of **13** (50 mg, 0.09 mmol) in MeOH (2 mL) was added 0.1 mL of hydrochloric acid (1M) at room temperature. The resulting mixture was refluxed for 10 min at 80°C, when TLC showed that no starting material remained. The reaction mixture was cooled and the solvent evaporated. The crude product was purified by flash chromatography to obtain **14** (28 mg) as a thick liquid in 75% yield. ^{1}H NMR **14** ($CDCl_3$) δ: 7.39-7.26 (m, 6H, C_6H & aromatic), 6.17 (t, 1H, J=6.9 Hz, 1′H), 5.45(2m, 1H, J_{HF}=48.0 Hz, N^3-C_5H), 4.68-4.57 (m, 1H, 3′H), 4.07-3.82 (m, 5H, N^3-C_1H, 4′ & 5′H), 2.58-2.40 (m, 2H, 2′H), 2.37-2.00 (m, 2H, N^3-C_4), 1.94 (d, 3H, J=0.9 Hz, CH_3), 1.90-1.64 (m, 2H, N^3-C_2H), 1.53-1.39 (m, 2H, N^3-C_3H). ^{19}F NMR **14** (δ): -174.98 (s, decoupled), -174.82 to -175.13 (m, coupled). High-resolution MS: M+Na, calculated 429.4395; found 429.4376.

Preparation of 3′,5′-O-bis-tetrahydropyranyl-N^3-(5-phenyl-5-chloropentyl)thymidine: 15

To a solution of **12** (100 mg, 0.17 mmol) in CH_2Cl_2 (4 mL) under argon, triethylamine (239 μL, 1.7 mmol) was added, followed by the addition of DMAP (6 mg, 0.05 mmol). The reaction mixture was cooled to 0°C and then MsCl (27 μL, 0.35 mmol) was added. The reaction mixture was warmed to room temperature, stirred and refluxed at 50°C for 6 h, when TLC showed that no starting material remained. The solvent was evaporated under vacuum, the residue was dissolved in CH_2Cl_2 (15 mL) and the solution was washed with H_2O (3x15 mL). The organic phase was dried ($MgSO_4$), evaporated to dryness, and purified on a silica gel column. Appropriate fractions were combined and evaporated to produce 61 mg of **23** in 60% yield. ^{1}H NMR **15** ($CDCl_3$) δ: 7.64, 7.58, 7.54, 7.52 (4d, 1H, J=1.2 Hz, C_6H), 7.42-7.28 (m, 5H, aromatic), 6.45-6.33 (m, 1H, 1′H), 4.87 (t, 1H, J=7.5 Hz, N^3-C_5H), 4.77-3.48 (m, 12H, N^3-C_1H, THP & 3′-5′H), 2.59-2.25 (m, 2H, 2′H), 2.24-2.00 (m, 2H, N^3-C_4H), 1.95, 1.92 (2s, 3H, CH_3), 1.89-1.45 (m, 16H, N^3-$C_{2,3}H$ &THP). MS: M+1, calculated 592.14; found 592.22.

Preparation of N^3-(5-phenyl-5-chloropentyl)thymidine: 16

To a solution of **15** (50 mg) in MeOH (3 mL) was added aqueous HCl (1M, 0.05 mL) and mixture was refluxed for 5 min. The solvent was evaporated, and the crude product was purified by flash chromatography to obtain **16** (30 mg) as a gummy material in 83% yield. ^{1}H NMR **16** ($CDCl_3$) δ: 7.41 (s, 1H, C_6H), 7.35-7.30 (m, 5H, aromatic), 6.19 (t, 1H, J=7.2 Hz, 1′H), 4.85 (t, 1H, J=7.5 Hz, N^3-C_5H), 4.63-4.54 (m, 1H, 3′H), 4.03-3.78 (m, 5H, N^3-C_1H, 4′H & 5′H), 2.54-2.38 (m, 2H, 2′H), 2.36-1.99 (m, 2H, N^3-C_4H), 1.94 (s, 3H, CH_3), 1.78-1.60 (m, 2H, N^3-C_2H), 1.51-1.40 (m, 2H, N^3-C_3H). High resolution MS: M+Na, calculated 445.8873; found 445.8861.

Preparation of 3′,5′-O-bis-tetrahydropyranyl-N^3-(5-phenyl-5-bromopentyl)thymidine: 17

3′,5′-O-bis-Tetrahydropyranyl-N^3-(5-phenyl-5-hydroxypentyl)thymidine (**12**) (130 mg, 0.23 mmol) was dissolved in

CH_2Cl_2 (4 mL) under argon, triphenylphosphine (66 mg, 0.25 mmol) was added, followed by the addition of 2,3-dichloro-5,6-dicyanobenzoquinone (57 mg, 0.25 mmol). Tetrabutylammonium bromide (81 mg, 0.25 mmol) was added into the reaction mixture and stirred for 3 h at room temperature, when TLC showed that no starting material remained. The solvent was evaporated under vacuum, the residue was dissolved in CH_2Cl_2 (20 mL), and the solution was washed with H_2O (3x20 mL). The organic phase was dried ($MgSO_4$), filtered and evaporated to dryness, and purified on a silica gel column. Appropriate fractions were combined and evaporated to produce 85 mg of **17** in 58% yield. ^{1}H NMR **17** ($CDCl_3$) δ: 7.68-7.52 (m, 1H, C_6H), 7.45-7.24 (m, 5H, aromatic), 6.45-6.34 (m, 1H, 1′H), 4.97 (t, 1H, J=7.5 Hz, N^3-C_5H), 4.75-3.50 (m, 12H, N^3-C_1H, THP & 3′-5′H), 2.60-2.26 (m, 2H, 2′H), 2.25-2.01 (m, 2H, N^3-C_4H), 1.96, 1.92 (2d, 3H, J=0.9 Hz, CH_3), 1.88-1.46 (m, 16H, N^3-$C_{2,3}$H &THP). MS: M+1, calculated 636.59, found 636.73.

Preparation of N^3-(5-phenyl-5-bromopentyl)thymidine: 18

The method of preparation of this compound was the same as described above for **14** and **16**. Briefly, **17** was hydrolyzed, and the crude product was purified by flash chromatography to obtain **18** as foam in 80% yield. ^{1}H NMR **18** ($CDCl_3$) δ: 7.44 (s, 1H, C_6H), 7.32-7.27 (m, 5H, aromatic), 6.16 (t, 1H, J=6.9 Hz, 1′H), 4.92 (t, 1H, J=7.5 Hz, N^3-C_5H), 4.64-4.55 (m, 1H, 3′H), 4.00-3.76 (m, 5H, N^3-C_1H, 4′ & 5′H), 2.56-2.39 (m, 2H, 2′H), 2.34-2.00 (m, 2H, N^3-C_4), 1.95 (s, 3H, CH_3), 1.88-1.57 (m, 2H, N^3-C_2H), 1.52-1.42 (m, 2H, N^3-C_3H). High-resolution MS: M+Na, calculated 490.3381; found 490.3396.

Preparation of 4-(4-Iodophenyl)-1-butanol: 20

Ethyl-4-(4-iodophenyl)butyrate (**19**) (5.0 g, 16.45 mmol) was dissolved in ether (Et_2O, 30 mL) and added to a slurry of $LiAlH_4$ (0.785 g, 20.73 mmol) in ether (30 mL) at 0°C. The reaction mixture was stirred for 16 h, during which time it was warmed to room temperature. After cooling to 0°C, the reaction was quenched by slow addition of MeOH and then diluted with ether. The mixture was washed with hydrochloric acid (2M, 40 mL) and then brine. The combined aqueous phases were extracted with ethyl acetate (EtOAc). The organic phases were then combined, dried (Na_2SO_4), and evaporated to give **20** as colorless oil (3.5 g) in 80% yield. ^{1}H NMR **20** ($CDCl_3$) δ: 7.57 (d, 2H, J=8.4 Hz, aromatic), 6.92 (d, 2H, J=8.4 Hz, aromatic), 3.62 (t, 2H, J=6.6 Hz, C_1H), 2.57 (t, 2H, J=7.2 Hz, C_4H), 1.64 (m, 2H, C_2H), 1.56 (m, 2H, C_3H). MS: M+1 calculated 277.00; found 277.20.

Preparation of 4-(4-Iodophenyl)-1-bromobutane: 21

To a solution of **20** (1.35 g, 4.9 mmol) and carbon tetrabromide (CBr_4, 1.94 g, 5.84 mmol) in CH_2Cl_2 (16 mL) under nitrogen was added slowly a solution of triphenyl phosphine (PPh_3) (1.57 g, 5.84 mmol) in CH_2Cl_2 (4 mL), and the mixture was stirred for 3 h at room temperature. The solvent was evaporated and the oily mixture was purified by flash chromatography (eluent hexane/EtOAc 8/2) to give **21** (1.13 g) in 68% yield. ^{1}H NMR **21** ($CDCl_3$) δ: 7.63 (m, 2H, aromatic), 6.95 (m, 2H, aromatic), 3.44 (t, 2H, J=7.2 Hz, C_1H), 2.61 (t, 2H, J=7.8 Hz, C_4H), 1.89 (m, 2H, C_2H), 1.78 (m, 2H, C_3H). MS: M+1, calculated 338.91; found 339.00.

Preparation of N^3-[4-(4-iodophenyl)-1-butyl]thymidine, N^3-(3-phenyl-1-propyl)thymidine and N^3-(5-phenyl-1-pentyl) thymidine: 24, 25 and 26

The method used to prepare **24**, **25** and **26** was similar, and a representative procedure is described. To a solution of thymidine (242 mg, 1mmol) in acetone/DMF (10 mL) (1:1), were added K_2CO_3 (109 mg, 1.1 mmol) and **21** (678 mg, 2 mmol). The reaction mixture was refluxed for 16 h. After evaporation of the solvent, the residue was dissolved in ethyl acetate (30 mL), washed with water (2x 20 mL), dried (Na_2SO_4), filtered and evaporated to dryness. The residue was purified by flash chromatography (eluent CH_2Cl_2/MeOH 9/1) to give **24** (305 mg) as a foam in 61% yield. ^{1}H NMR **24** ($CDCl_3$) δ: 7.56 (d, 2H, J=8.4 Hz, aromatic), 7.36 (s, 1H, C_6H), 6.92 (d, 2H, J=8.4 Hz, aromatic), 6.19 (t, 1H, J=6.6 Hz, 1′H), 4.56-4.54 (m, 1H, 3′H), 4.00 (q, 1H, J=3.0 Hz, 4′H), 3.96-3.91 (m, 2H, N^3-C_1H), 3.86 (dd, AB, 2H, J=3.0, 12.0 Hz 5′H), 2.58 (t, 2H, J=6.6 Hz, C_4H), 2.40-2.30 (m, 2H, 2′H), 1.93 (s, 3H, CH_3), 1.62 (m, 4H, N^3-$C_{2,3}$H). MS: M+1, calculated 501.18; found 501.30.

Compound (**25**) was obtained as a white solid in 64% yield. MP: 162-163.5. ^{1}H NMR **25** (DMSO-D_6) δ: 7.73 (s, 1H, C_6H), 7.26 (t, 2H, J=7.8 Hz, aromatic), 7.20 (d, 2H, J=7.2 Hz, aromatic), 7.16 (t, 1H, J=7.2 Hz, aromatic), 6.20 (t, 1H, J=6.6 Hz, 1′H), 5.23 (d, 1H, J=3.6 Hz, 3′OH), 5.02 (t, 1H. J=4.2 Hz, 4′OH), 4.24 (m, 1H, 3′H), 3.83 (t, 2H, J=7.2 Hz, N^3-C_1H), 3.77 (q, 1H, J=3.6 Hz, 4′H), 3.63-3.53 (m, AB, 2H, 5′H), 2.60 (t, 2H, J=7.2 Hz, N^3-C_3H), 2.09 (2d, 2H, J=4.2 Hz, 2′H), 1.86-1.80 (m, 2H, N^3-C_2H), 1.81 (s, 3H, CH_3); MS: M+1, calculated 361.16; found 361.25.

Compound (**26**) was obtained as a gummy material in 58% yield. ^{1}H NMR **26** ($CDCl_3$) δ: 7.40 (s, 1H, C_6H), 7.26 (t, 2H, J=7.8 Hz, aromatic), 7.17-7.14 (m, 3H, aromatic), 6.20 (t, 1H, J=6.6 Hz, 1′H), 4.52-4.50 (m, 1H, 3′H), 3.98 (q, 1H, J= 3.0 Hz, 4′H), 3.92-3.79 (m, 4H, N^3-C_1H & 5′H), 2.60 (t, 2H, J=7.8 Hz, N^3-C_5H), 2.33-2.30 (m, 2H, 2′H), 1.91 (s, 3H, CH_3), 1.67-1.60 (m, 4H, N^3-$C_{2,4}$H), 1.40-1.37 (m, 2H, N^3-C_3H). MS: M+1, calculated 389.20; found 389.30.

Phosphoryl Transfer Assay with Recombinant TK1:

Thymidine and the N^3-substituted nucleoside analogues were dissolved in DMSO to produce stock solutions of various concentrations (30-140 μM). The assays were carried out as described previously [34] with minor modifications. In TK1 assays, the reaction mixture contained 10 μM nucleoside, 100 μM ATP (1 μCi of [γ-^{32}P]ATP) (Amersham), 50 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 125 mM KCl, 10 mM DTT, and 0.5 mg/mL bovine serum albumin (BSA). In all reactions, the final concentration of DMSO was set to 1%. The reaction mixture was incubated at 37°C for 20 min in the presence of 100 ng of enzyme. Following the incubation period, the enzyme was inactivated by heating the reaction mixture for 2 min at 95°C. The reaction mixture was centrifuged and 1 μL sample portions were spotted on PEI-cellulose TLC plates (Merck).

The TLC plates were developed in a solvent system containing isobutyric acid/ammonium hydroxide/water (66/1/33). The TLC plates were then scanned on a TLC scanner (Bioscan, Washington DC) for radioactivity. Alternatively,

the crude reaction mixture was analyzed by HPLC as described earlier [18]. Briefly, 10 μL of each reaction mixture was injected to the HPLC and 15-sec fractions were collected. Radioactivity in each fraction was measured in a scintillation counter and plotted with the fraction number to generate radiochromatograms. A standard solution of the mixture of thymidine phosphates was co-injected to establish the retention times of cold phosphates. The percentage of monophosphate of the nucleoside analogue formed by this enzymatic transfer of phosphate from ATP was calculated on the basis of the formation of thymidine monophosphate.

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