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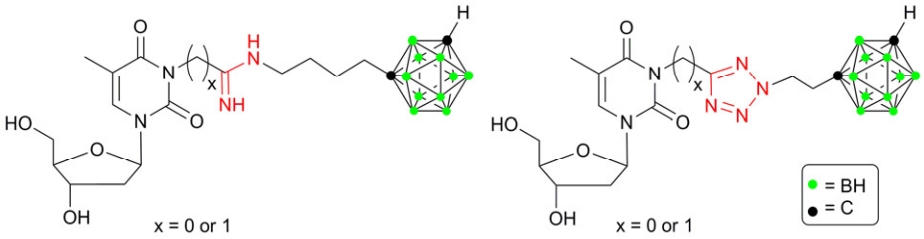
Highlights

- Four libraries of N3-carboranyl thymidine analogues (3CTAs) were synthesized.
- 3CTAs were evaluated for improved binding to human thymidine kinase 1.
- Selected 3CTAs were subjected to in-depth enzyme kinetics studies (rk_{cat}/K_{m}).
- Solubility studies of 3CTAs were conducted in PBS at pH 7.4.



Synthesis of N3-Substituted Carboranyl Thymidine Bioconjugates and their Evaluation as Substrates of Recombinant Human Thymidine Kinase 1

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Abbreviations: ATP, Adenosine triphosphate; 3CTAs, 3-Carboranyl Thymidine Analogues; BNCT, Boron Neutron Capture Therapy; PBS, phosphate buffered saline; ^{10}B, boron-10; hTK1, human thymidine kinase 1; KMT, Kinase Mediated Trapping; DMSO, dimethyl sulfoxide; dThd, thymidine; DMF, dimethyl formamide; TBDMS, *tert*.-butyldimethylsilyl; TBAF, tetrabutylammonium fluoride; HR-MS, High Resolution Mass Spectra; LR-MS, Low Resolution Mass Spectra: ESI-MS, Electrospray Ionization Mass Spectra; AUCs, areas under the curves; PTA, Phosphorylation Transfer Assay; BSA, Bovine serum albumin; dTMP, thymidine monophosphate.

Abstract

Four different libraries of overall twenty three N3-substituted thymidine (dThd) analogues, including eleven 3-carboranyl thymidine analogues (3CTAs), were synthesized. The latter are potential agents for Boron Neutron Capture Therapy (BNCT) of cancer. Linker between the dThd scaffold and the *m*-carborane cluster at the N3-position of the 3CTAs contained amidinyl-(**3e** and **3f**), guanidyl-(**7e-7g**), tetrazolylmethyl-(**9b1/2-9d1/2)**, or tetrazolyl groups (**11b1/2-11d1/2)** to improve human thymidine kinase 1 (hTK1) substrate characteristics and water solubilities compared with 1st generation 3CTAs, such as **N5** and **N5-2OH**. The amidinyl- and guanidyl-type N3-substitued dThd analogues (**3a**-**3f** and **7a**-**7g**) had hTK1 phosphorylation rates of <30% relative to that of dThd, the endogenous hTK1 substrate, whereas the tetrazolyl-type N3-substitued dThd analogues (**9a, 9b1/2-9d1/2** and **11a, 11b1/2-11d1/2**) had relative phosphorylation rates (rPRs) of >40%. Compounds **9a, 9b1/2-9d1/2** and **11a, 11b1/2-11d1/2** were subjected to in-depth enzyme kinetics studies and the obtained rk_{cat}/K_m (k_{cat}/K_m relative to that of dThd) ranged from 2.5-26%. The tetrazolyl-type N3-substitued dThd analogues **9b1/2** and **11d1/2** were the best substrates of hTK1 with rPRs of 52.4% and 42.5% and rk_{cat}/K_m values of 14.9% and 19.7% respectively. In comparison, the rPR and rk_{cat}/K_m values of **N5-2OH** in this specific study were 41.5% and 10.8%, respectively. Compounds **3e** and **3f** were >1,900 and >1,500 times, respectively, better soluble in PBS (pH 7.4) than **N5-2OH** whereas solubilities for **9b1/2-9d1/2** and **11b1/2-11d1/2** were only 1.3 – 13 times better.

Keywords:

Thymidine, *m*-Carborane, Boron Neutron Capture Therapy (BNCT), human Thymidine Kinase 1 (hTK1), Thymidine conjugates.

1. Introduction

Thymidine (dThd) bioconjugates with carborane clusters (3-carboranyl dThd analogues or 3CTAs) have been studied extensively for Boron Neutron Capture Therapy (BNCT), which is a radio-chemotherapeutic modality for cancer treatment [1,2]. These bioconjugates were designed for selective uptake and retention in tumor cells by kinase mediated trapping (KMT) through 5′-monophosphorylation by human thymidine kinase 1 (hTK1), an enzyme of the salvage pathways of DNA precursor synthesis with high activity levels only in rapidly proliferating cells [1,3]. So far, KMT has been employed primarily in positron emission tomography (PET) of tumors using 3′-deoxy-3′-[^{18}F]fluorothymidine [^{18}F-FLT] as the imaging agent [4].

First generation 3CTAs, such as **N5** and **N5-2OH** (**Fig. 1**), were found to be substrates of hTK1 in enzyme kinetic studies [5-9]. The *in vitro* uptake of **N5** and **N5-2OH** in L929 TK (+) cells was 5-10 fold higher than in L929 TK (-) cells [6]. Biodistribution studies with **N5-2OH** in rats bearing intracranial RG2 glioma resulted in 10 fold higher boron concentrations in the tumor than in normal brain (27.6 µg B/g tumor vs. 2.6 µg B/g tumor) [8]. BNCT with rats bearing intracranial RG2 glioma that had received **N5-2OH** prior to irradiation increased survival times by a factor two compared with BNCT of rats bearing RG2 glioma that had not received **N5-2OH** [8].

These biological studies clearly established the therapeutic potential for 3CTAs. On the other hand, the same studies [5-9] revealed two shortcomings of **N5** and **N5-2OH**: 1) The lipophilic nature of **N5-2OH** caused low water-solubility, which necessitated the use of organic solvents, such as DMSO, to solubilize the agent for biological studies. 2) Compared with endogenous dThd, **N5** and **N5-2OH** show suboptimal hTK1 substrate characteristics.

X-ray crystallographic data with various TK1-like enzymes indicated that hydrogen bonds between the N3-H, C2=O, and C4=O of dThd and the amide backbone of the so-called "lasso-loop" [10,11] of the enzymes are crucial for substrate binding [10-16]. In the case of hTK1 co-crystallized with dThd triphosphate, N3-H and C2=O of the dThd scaffold interacted with Val 172 and Val 174 of the lasso-loop [10,13]. Similar studies with *Bacillus anthracis* TK (*Ba*TK) indicated a hydrogen bond network between Tyr 179 and Arg 157 of the lasso-loop and N3-H and C4=O of dThd [1]. In the case of *Ureaplasma urealyticum* TK (*Uu*TK), hydrogen bonds occurred between N3-H and C4=O of dThd and Ile 178 and Lys 180 of the lasso-loop, respectively [10,11]. In all of these cases, N3-H and C2=O and/or C4=O acted as proton donors and acceptors, respectively. Sequra-Peña *et al.* studied in depth the structural changes in *Thermotoga maritima* thymidine kinase (*Tm*TK) as a result of dThd and ATP binding [14]. Apparently, interactions similar to those discussed above contributed to dramatic conformational changes in the lasso-loop resulting in the conversion of an open (apo) form to a closed (holo) form of the enzyme, which locks dThd tightly into the substrate binding pocket [12,14]. In the cases of **N5** and **N5-2OH**, the N3-proton is replaced by a bulky carboranyl alkyl chain. Thus, hydrogen bonds crucial for the induction of closed

conformations of TK1-like enzymes are missing [1]. We hypothesize that this may limit the abilities of **N5** and **N5-2OH** to interact with the substrate-binding site of the enzyme. In addition, computational studies indicated that **N5-2OH** cannot be docked effectively into the substrate binding pockets of holo TK1-like enzymes [5] whereas docking was possible in the case of an apo form. This suggests that the presence of the bulky carborane cluster may also interfere with the induction of a completely closed enzyme confirmation. Since the final binding confirmation of TK1-like enzymes with **N5-2OH** and other 3CTAs is unknown, computer-aided structural design is not an effective tool to support structure-activity-relationship (SAR) studies in pursuit of improved 3CTAs.

A bulky carboranyl substituent at N3 is the trademark of any 3CTA and this cannot be changed. On the other hand, it is conceivable that the missing hydrogen bond interaction between NH-3 and C=O, in part, can be compensated by introducing functional groups in the vicinity of N3 of the 3CTA scaffold having the ability to re-introduce hydrogen bonding. Thus, this paper discusses the synthesis, hTK1 substrate characteristics, and the results of water solubility measurements of four novel classes of N3-substituted dThd analogues: N3-amidinyl- (**3a**-**3f**), N3-guanidyl- (**7a**-**7g)**, N3-(1-tetrazolylmethyl)- (**9a, 9b1/2**-**9d1/2**), and N3-tetrazolyl-dThd analogues (**11a, 11b1/2**-**11d1/2**) [**Schemes 1**-**4**]. The amidinyl- and guanidyl functions in the spacer between the dThd scaffold and the *m*-carborane cage of target compounds **3a-3f** and **7a-7g** serve as proton donors. They may restore hydrogen bond interactions with proton accepting atoms in amino acid side chains as well as in the peptide back bone of the lasso loop of hTK1 thereby inducing a more closed enzyme conformation. This, in turn, may render these types of dThd analogues more tightly bound to the active site. As a result, target

compounds **3a-3f** and **7a-7g** may have better enzyme substrate characteristics in comparison with **N5-2OH**. In addition, the basic nitrogen atoms in these structures should be protonated under physiological conditions, which will improve their water-solubilities. In contrast to the amidinyl- and guanidyl groups in target compounds **3a-3f** and **7a-7g**, the tetrazolyl moieties in target compounds **9a, 9b1/2-9d1/2** and **11a, 11b1/2-11d1/2** have proton accepting ability only. Nevertheless, they may still interact with various proton donating atoms in amino acid side chains (e.g. Arg 165, Lys 170, Lys 180) and in the peptide back bone of the lasso-loop thereby facilitating a closed enzyme conformation. Lesnikowski *et al.* recently described the synthesis of several N3-substituted triazolylcarboranyl dThd analogues [17,18] that may have similar TK1-binding properties than the tetrazolyl-type N3-substitued dThd analogues (**9a, 9b1/2-9d1/2** and **11a, 11b1/2-11d1/2**) described in this paper.

2. Results and discussion

2.1. Chemistry

Four different libraries of N3-substituted dThd analogues were synthesized in two general steps. In the first step, 3-(cyanomethyl)thymidine (**2**, **Scheme 1**) and 3',5'-(di-*tert*.-butyldimethylsilyl)-3-cyanothymidine (**5**, **Scheme 2**) were synthesized by the reaction of dThd and 3′,5′-di-TBDMS-protected dThd, with iodoactonitrile and cyanogen bromide, respectively. In the second step, these reactive nitriles were treated with various *m*-carboranylalkyl- [19], alkyl-, and arylamines to yield amidinyl- and guanidyl-type N3-substitued dThd analogues (**3a-3f** and **7a-7g**, **Schemes 1** and **2**). Compounds **2** and **5** were also treated with sodium azide to produce the tetrazolyl-type dThd analogues **8** and

10 (**Schemes 3** and **4**), which were subsequently treated with various *m*-carboranylalkyl-[19] and alkyliodides to yield the tetrazolyl-type target compounds **9a, 9b1/2-9d1/2** and **11a, 11b1/2-11d1/2** (**Schemes 3** and **4**). *m-closo*-Carborane was used for the synthesis of all carboranyl target compounds (**3e-3f**, **7e-7g**, **9b1/2-9d1/2** and **11b1/2-11d1/2**) because it is more resistant to basic degradation than *o-closo*-carborane [19], which could occur particularly during the synthesis and biological evaluation of the amidine- and guanidine containing compounds **3e**, **3f** and **7e-7g**.

3-(Cyanomethyl)thymidine (**2**) was synthesized in 78% yield by selective *N*-alkylation at the 3-position of dThd with iodoacetonitrile (**Scheme 1**) in presence of potassium carbonate in DMF/acetone (1/1, v/v) at 60 °C for 6 h. Compound **2** was purified by silica gel column chromatography. For the synthesis of the non-carboranyl amidine-type dThd analogues **3a-3d**, compound **2** and alkyl- or arylamines were heated in a closed pressure reaction vessel (Quark Glass) in the presence of hexafluoroisopropanol (HFIP) overnight at 95 °C. The use of HFIP for this type of reaction has been previously reported by Snider and O'Hare [22]. The use of methanol, *tert.*-butanol, tetrahydrofuran, and acetonitrile as solvents did not lead to product formation in this study [22]. The reaction mixtures were added to water and extracted with ethyl acetate. The aqueous layers were separated, evaporated, and purified by RP-18 HPLC. Yields ranged from 24% to 81%.

For the synthesis of the *m*-carboranylalkyl target compounds **3e** and **3f**, the same method proved unsuccessful and a modified strategy was developed. Equimolar quantities of **2**, *m*-carboranyl alkyl amine [19], and 1 mL of HFIP were placed in a round bottom flask and heated overnight at 120 °C without condenser. Heating resulted in

complete solvent evaporation and formation of **3e** and **3f** in yields of 7% and 10%, respectively, after purification by RP-18 HPLC.

The synthesis of N3-guanidine-type dThd analogues (**7a-7g**, **Scheme 2**) was carried out with 3′,5′-di-TBDMS-protected dThd (**4**) [20] as the starting material, because initial attempts to treat dThd with cyanogen bromide in presence of potassium carbonate resulted in <5% yield of the desired product, 3-cyanothymidine. Reaction of **4** with cyanogen bromide in presence of triethylamine (TEA) in acetone at 0 °C gave 3',5'-(di-*tert*.-butyldimethylsilyl)-3-cyanothymidine (**5**) in 90% yield after column chromatographic purification [21].

Compounds **7a-7g** were synthesized by treating **5** with the amines shown in **Scheme 2** in presence of HFIP in a closed pressure reaction vessel (Quark Glass) [22]. The reactions of **5** with propyl- and butyl amine were carried out in an ice bath for 5 h to give **6a** and **6b**, respectively, in > 95% yield whereas phenyl- and benzyl amines required overnight reaction at room temperature to yield **6c** and **6d**, respectively, in > 90% yield. The reaction of 1-(2-aminoethyl)-*m*-carborane [19], 1-(3-aminopropyl)-*m*-carborane [19], and 1-(4-aminobutyl)-*m*-carborane [19] with **5** was carried out overnight at 95 °C to afford **6e**, **6f**, and **6g**, respectively, each in about 30-40% yield. Compounds **6a-6g** were dissolved in trifluoroacetic acid (TFA)/dichloromethane (10 %, v/v) and stirred at room temperature for 1 h producing **7a-7g** in 40-82% yield after purification by RP-18 HPLC. Both N3-amidinyl- (**3a-3f**) and guanidyl-type (**7a-7g**) dThd analogues were found to be unstable at room temperature (over several days) and were therefore stored at -5 °C. However, even under these storage conditions partial to complete degradation was observed after a ~ 1 year period.

The synthesis of tetrazolylmethyl- and tetrazolyl-type target compounds **9a, 9b1/2-9d1/2** and **11a, 11b1/2-11d1/2** is described in **Schemes 3** and **4**, respectively. These compounds were prepared using a click chemistry approach [23]. The reactive nitrile group of **2** was treated with sodium azide in the presence of zinc bromide ($ZnBr_2$) in a mixture of water and isopropanol (2:1, v/v) at 90 °C for three days to produce **8** in 90% yield (**Scheme 3**). In case of **5**, the same reaction conditions led to a mixture of 3-(tetrazol-5-yl)thymidine (**10**, **Scheme 4**) along with 3′- or 5′-mono-TBDMS and 3′,5′-(di-TBDMS)-3-(tetrazol-5-yl)thymidine. Deprotection of remaining TBDMS-groups was accomplished *in situ* by adding TFA in dichloromethane (10%, v/v) to the reaction mixture for 1 h at room temperature to give **10** in 44% overall yield (**Scheme 4**). Both **8** and **10** were purified by RP-18 HPLC.

Compound **8** and **10** were *N*-alkylated at the tetrazolyl ring with 1-iodopropane, 1-(2-iodoethyl)-*m*-carborane [19], 1-(3-iodopropyl)-*m*-carborane [19], and 1-(4-iodobutyl)-*m*-carborane [19] in the presence of potassium carbonate in a mixture of acetone and dimethylformamide (50%, v/v) at 60 °C overnight to produce target compounds **9a** and **11a** as well as **9b1/2-9d1/2** and **11b1/2-11d1/2** as isomeric (N^b-alkylated and N^c-alkylated) mixtures (**Schemes 3** and **4**). In the case of the propyl analogues **9a** and **11a** it was possible to isolate the N^c-isomers as pure compounds by RP-18 HPLC with 59 % to 71% yield, respectively. Unfortunately, it was not possible to separate the isomers of the carboranylalkyl derivatives **9b1/2-9d1/2** and **11b1/2-11d1/2**. Overall, the N^c-isomers were the dominating species in these mixtures with 70-95% (**Schemes 3** and **4**). The isomers were characterized by means of ^{1}H-NMR. The chemical shifts for the N^b-CH_2 protons ranged from 4.44-4.62 ppm, whereas those for the N^c-CH_2 protons ranged from

4.54-4.82 ppm. A downfield shift of 0.15-0.53 ppm for N^c-CH_2 protons vs. the N^b-CH_2 protons was also reported by Sveshnikov and Nelson [24] for various alkyltetrazoles. The observed N^c:N^b isomeric ratios were in accordance with those reported previously for other alkylation reactions at tetrazole rings [25]. In addition, ^{1}H-^{1}H NOESY NMR of the mixture of **9b1** and **9b2** showed a NOE cross-peak between the triplet for N^b-CH_2 (δ 4.52) and the singlet for N3-CH_2 (5.35 ppm). In contrast, no cross peak was observed between the signals of N^c-CH_2 and N3-CH_2 (see Supplementary material). This result was expected based on a shorter interproton distance between N^b-CH_2 and N3-CH_2 (2.5 Å) than between N^c-CH_2 and N3-CH_2 (4.9 Å) (calculated with Chem3D Pro [Chambridge Soft, Cambridge, MA, USA]). It unambiguously confirmed the isomer assignment.

2.2. Enzymatic studies

Compounds **3a**-**3f**, **7a**-**7g**, **8**, **9a, 9b1/2-9d1/2**, **10**, and **11a, 11b1/2-11d1/2** were evaluated in phosphoryl transfer assays (PTAs) with recombinant hTK1 as described previously with minor modifications [26-29]. Thymidine and **N5-2OH** were used as reference compounds. The obtained phosphorylation rates are expressed as percent of the phosphorylation rate of dThd, which was set to 100% (rPRs). Such comparison is used to give an estimation of the structure activity relationships of substrate analogues compared to the activity of the natural substrate, dThd. Analogues with relatively high PRs were further selected for in depths kinetic studies (apparent k_{cat}/K_m values) to determine the apparent catalytic efficiency of hTK1 for these substrates.

Most of the amidinyl-type dThd analogues (**3a**-**3f**) and all of the guanidyl-type dThd analogues (**7a**-**7g**) proved to be unstable under phosphoryl transfer assay conditions, as

evidenced by the production of phosphorylated side products. **Fig. 2A** shows the monophosphate formation of the carboranyl compounds of both libraries (**3e**, **3f**, **7e-7g**). For compounds **7e-7g**, additional spots are visible at the level of dThd monophosphate (dTMP). This indicates that these compounds may have been partially degraded to dThd during the assay, which was then converted to dTMP. A Low Resolution-Electron Spray Ionization (LR-ESI) mass spectra of an enzyme assay mixture containing compound **7f** and ^{31}P-ATP showed a signal for the monophosphate of **7f** (Calcd. for $[M\text{-}PO_3H_2+H]^+$: 549.3; Found: *m/z* 549.3) and a signal for dTMP (Calcd for $[M+H]^+$: 323.1; Found: *m/z* 323.1, see Supplementary material). The observed MS data support the formation of dTMP during the enzyme assay as a result of degradation processes. Enzyme assay mixtures containing compounds **3e** and **3f** did not appear to produce significant amounts of dTMP as a side product. However, it is conceivable that in the case of both compounds, dTMP may not be visible because of low concentrations

Compounds with alkylamidinyl- and alkylguanidyl substituents (**3a**, **3b**, **7a**, **7b**) had rPRs of 0-3%, whereas those with arylamidinyl- and arylguanidyl substituents (**3c**, **3d**, **7c**, **7d**) had slightly higher rPRs of 2-17% (**Table 1**). However, for compounds **3a**-**3d** and **7a**-**7d**, 20-100% of the formed phosphorylated material appeared to be the dTMP side product. Compounds with *m*-carboranylalkylamidinyl substituents **3e** and **3f** had rPRs of 3% and 6% whereas the *m*-carboranylalkylguanidyl compounds **7e**-**7g** had overall slightly higher rPRs with 16-27% but also showed the formation of the dTMP side product with rPRs of 6-12% (**Table 1** and **Fig. 2A**). Overall, the rPRs of both the amidinyl-type and the guanidyl-type N3-substitued dThd analogues were lower than that of **N5-2OH** (rPR of 46%).

In contrast to the amidinyl- (**3a-3f**) and guanidyl-type (**7a-7g**) dThd analogues, the tetrazolylmethyl-type (**8**, **9a, 9b1/2-9d1/2**) and the tetrazolyl-type dThd analogues (**11a, 11b1/2-11d1/2**) were stable under phosphoryl transfer assay conditions (**Table 1**, **Fig. 2B** and **Fig. 2C**). With the exception of compound **10**, all tetrazolyl-type dThd analogues displayed relatively higher rPRs with 40-64%. The signals for monophosphate product of **10** may actually overlap with ATP related spots. The tetrazolylmethyl-type dThd analogues with propyl- and butyl spacer between carborane and tetrazole (**9c1/2**, **9d1/2**) appeared to be somewhat better phosphorylated than their tetrazolyl-type counterparts (**11c1/2**, **11d1/2**) (58-64% vs. 42-43%).

The tetrazolyl-type dThd analogues **9a, 9b1/2-9d1/2** and **11a, 11b1/2-11d1/2** were selected for in depths kinetic studies (apparent k_{cat}/K_m values) based on their rPRs, which were higher than that of **N5-2OH**. Both dThd and **N5-2OH** were used as reference compounds. These studies were conducted with varying substrate concentrations (0.4-300 µM) (see Experimental Section for details). The rk_{cat}/K_m value for **N5-2OH** using the current recombinant hTK1 preparation with a specific activity of 441 nmol dThd monophosphate formation per min and mg of enzyme was lower compared with the value obtained with a different enzyme preparation used in a previous study (10.8% vs 35.8%) [9]. Narayanasamy et al. [26] reported a specific activity of 640 nmol dThd monophosphate formation per min and mg of enzyme for a different recombinant hTK1 used in a previous study with 3CTAs. This suggests that specific activities between enzyme preparations vary significantly and that this, in turn, may cause variations in the rk_{cat}/K_m values of 3CTAs. The rk_{cat}/K_m values for compound **9a**, **9c1/2**, **9d1/2**, and **11b1/2** were comparable with that of **N5-2OH** (7.6-12.9% vs. 10.8%). Compounds **9b1/2**

(14.9%), **11a** (26.0%) and **11d1/2** (19.7%) had notably higher rk_{cat}/K_m values indicating that these compounds are better substrates of hTK1 than **N5-2OH**.

Based on the isomeric composition of the tetrazoyl target compounds **9a, 9b1/2-9d1/2** and **11a, 11b1/2-11d1/2** (**Schemes** and **4**), it is reasonable to assume that their observed enzymatic activities are primarily related to the N^c-isomers.

2.3. Solubility studies

The solubilities of carboranyl compounds that appeared to be stable during enzyme assays (**3e**, **3f**, **9b1/2-9d1/2, and 11b1/2-11d1/2**) were determined in phosphate-buffered saline (PBS) at 25 °C in comparison with **N5-2OH** using thermodynamic solubility measurement assay conditions (Table 3) [27,28]. The carboranylethyl dThd analogue **3e** and its propyl homologue **3f** were > 1900 and >1500 times, respectively, better soluble in PBS than **N5-2OH**. The solubility patterns for the tetrazolylmethyl-(**9b1/2-9d1/2**) and tetrazolyl- (**11b1/2-11d1/2**) dThd analogues decreased with increasing chain length. Compounds **9b1/2-9d1/2** were ~3-13 times better soluble in PBS than **N5-2OH** whereas compounds **11b1/2** and **11c1/2** were ~2-4 times better soluble. The solubility profile of **11d1/2** was similar to that of **N5-2OH**. Overall, the tetrazolylmethyl derivatives were slightly better soluble than their corresponding tetrazolyl counterparts.

3. Summary and Conclusions

In summary, several N3-substituted dThd analogues containing amidinyl-(**3a-3f**), guanidyl-(**7a-7g**), tetrazolylmethyl-(**8**, **9a, 9b1/2-9d1/2)**, tetrazolyl groups (**10**, **11a, 11b1/2-11d1/2)** were successfully synthesized. The amidinyl- and guanidyl-type dThd

analogues (**3a**-**3f** and **7a**-**7g**) did not possess the necessary stability under aqueous conditions. The excellent solubilities of **3e** and **3f** in PBS were expected because of the presence of basic amidinyl groups in these structures, which should be protonated at physiological pH (7.4). Unfortunately, the solubilities of the tetrazolyl-type dThd analogues in PBS were only moderately better than that of **N5-2OH**. The tetrazolylmethyl- (**8**, **9a, 9b1/2-9d1/2**) and tetrazolyl-type dThd analogues (**10**, **11a, 11b1/2-11d1/2**) had comparable or somewhat higher rPRs (40-64%) than that of **N5-2OH** whereas those of the amidine- (**3a**-**3f**) and guanidine-type (**7a**-**7g**) dThd analogues had lower rPRs with 2-12% and 0-27%, respectively. The rk_{cat}/K_m values for the tetrazolylcarboranyl dThd analogues with relatively high rPRs (**9a, 9b1/2-9d1/2**, **11a, 11b1/2-11d1/2)** ranged from 7.6-26% in comparison to **N5-2OH** (10.8%). Compounds **11d1/2** (rk_{cat}/K_m = 19.7%) and **9b1/2** (rk_{cat}/K_m = 14.9%) were ~1.8 and ~1.4 times, respectively, better substrates of hTK1 than **N5-2OH**.

The rk_{cat}/K_m values obtained for compounds **9b1/2** and **11d1/2** indicate that proton accepting groups in the spacer between carborane cluster and dThd scaffold have the potential to improve hTK1 substrate characteristics. In addition, basic functions in the spacer seem to enhance aqueous solubility, as demonstrated at the example of compounds **3e** and **3f**. Unfortunately, the validity of enzymatic data obtained for amidinyl- and guanidyl-type dThd analogues (**3a**-**3f** and **7a**-**7g**) is limited due to lack of stability under aqueous and routine storage conditions. Preliminary studies in our laboratories indicated that inserting more than two methylene groups between the dThd scaffold and basic amino group containing functions in the spacer prevents decomposition under aqueous conditions. As discussed in the introduction, a major shortcoming is lack of detailed

structural information that can assist in delineating the basic molecular interactions between hTK1 and 3CTAs.

4. Experimental protocol

4.1. Chemistry

^{1}H- and ^{13}C NMR spectra were obtained on a Bruker DRX 400 at The Ohio State University College of Pharmacy (400 MHz for ^{1}H and 100 MHz for ^{13}C and 128 MHz for ^{11}B). ^{1}H-^{1}H NOESY NMR was obtained on a Bruker DRX 800 at The Ohio State University Campus Chemical Instrument Center. Chemical shifts (δ) are reported in ppm from internal deuterated chloroform, acetone, and methanol. Coupling constants are reported in Hz. ^{13}C NMR spectra are fully decoupled. Low- and High Resolution–Electrospray ionization (LR/HR-ESI) mass spectra were obtained on a Micromass LCT spectrometer at The Ohio State University Campus Chemical Instrumentation Center, Columbus, OH. For all carborane-containing compounds, the mass of the most intensive peak of the isotopic pattern was reported (80% boron-11 to 20% boron-10 distribution). Measured patterns agreed with calculated patterns. Silica gel 60 (0.063-0.200 mm) used for gravity column chromatography and silica gel 60 (0.015-0.049 mm) used for flash column chromatography were purchased from Dynamic Adsorbents Inc. (3280 Peachtree Corners Circle, E, Norcross, GA 30092, USA). Reagent-grade solvents were used for column chromatography. Precoated glass-backed TLC plates with silica gel 60 F254 (0.25-mm layer thickness) from Dynamic Adsorbents (Norcross, GA) were used for TLC. General compound visualization for TLC was achieved by UV light. Carborane-containing compounds were selectively visualized by spraying the plate with a 0.06%

$PdCl_2$/1% HCl solution and heating at 120 °C, which caused the slow (15-45 s) formation of a gray spot due to the reduction of Pd^{2+} to Pd0. Anhydrous solvents, such as dichloromethane, were purchased directly either from Acros Organics (Morris Plains, NJ) or from Sigma Aldrich (Milwaukee, WI). Pressure reaction vessels were purchased from Quark Glass (NJ, USA). Other chemicals and solvents were purchased from standard commercial suppliers. Tetrahydrofuran (THF) and benzene were distilled from sodium and benzophenone indicator under argon. *m*-Carborane was purchased from Katchem Ltd. (Prague, Czech Republic). Cyanogen bromide (purchased from Sigma Aldrich) is a highly toxic compound. The Material Safety Data Sheet (MSDS) should be consulted before usage. [γ-^{32}P]ATP (specific activity: 3000Ci/mmol) and [methyl-^{3}H]dThd (specific activity: 83.7 Ci/mmol) were obtained from Perkin Elmer, Waltham, MA. Unless specified otherwise, all reactions were carried out under argon atmosphere. Preparative HPLC was performed with a Gemini 5µ C18 column (21.20 mm x 250 mm, 5 µm particle size) supplied by Phenomenex Inc. CA, USA on a Hitachi HPLC system (L-2130) with a Windows based data acquisition and Hitachi Diode array detector (L-2455). Purification was accomplished using a water (0.1% TFA)/methanol (0.1% TFA) gradient at 7 mL/min flow rate (method: 100:0 to 50:50 over 70 min followed by 50:50 to 0:100 over 40 min). Analytical HPLC was carried out with a Gemini 5µ C18 110A Column (250 x 4.6 mm) supplied by Phenomenex Inc. CA, USA using the HPLC system above. Two different gradients [water (0.1% TFA)/acetonitrile (0.1% TFA) and water (0.1% TFA)/methanol (0.1% TFA)] were used at 1 mL/min flow rate (method: 100:0 to 75:25 over 20 min followed by 75:25 to 100:0 over 10 min). See Supplementary material for more details on compound purification and analysis.

*4.1.1. 3-(Cyanomethyl)thymidine (**2**).* Thymidine (10 g, 41.3 mmol) and potassium carbonate (17 g, 123 mmol) were dissolved in acetone/DMF (100 mL, 1/1, v/v) and iodoacetonitile (6.5 mL, 90 mmol) was added. The reaction mixture was refluxed for 6 h, then added to water (750 mL), and extracted with ethyl acetate (250 mL × 4). The organic layer was separated, dried using anhydrous magnesium sulfate (100 g), evaporated under reduced pressure, and purified by silica gel column chromatography using dichloromethane and methanol as the solvent system. The compound was eluted at 4% methanol. R_f = 0.70 (10% methanol in dichloromethane), yield = 9.1 g, 78% as yellow solid, (m.p. 52 °C); ^{1}H NMR (400 MHz, CD_3OD, δ ppm): 7.86 (s, 1H, H-6), 6.25 (t, *J* = 6.6 Hz, 1H, 1'H), 4.80 (s, 2H, C*H*$_2$-CN), 4.33-4.41 (m, 1H, 3'H), 3.87-3.95 (m, 1H, 4'H), 3.78 (dd, *J* = 2.8 and 12.0 Hz, 2H, 5''H), 3.70 (dd, *J* = 3.1 and 12.0 Hz, 2H, 5'H), 2.23-2.33 (m, 1H, 2''H), 2.12-2.22 (m, 1H, 2'H), 1.89 (s, 3H, 5-C*H*$_3$). ^{13}C NMR (CD_3OD, 100 MHz, δ ppm): 163.29 (C-4 C=O), 150.62 (C-2 C=O), 136.77 (C-6), 115.46 (*C*N), 110.30 (C-5), 88.27 (C-1'), 86.79 (C-4'), 71.40 (C-3'), 62.16 (C-5'), 41.09 (C-2'), 28.92 (N3-*C*H$_2$-CN), 13.05 (5-*C*H$_3$); HR-MS (ESI-MS) for $C_{12}H_{15}N_3NaO_5$. Calcd: 304.0909. Found: *m/z* 304.0888 $(M+Na)^+$.

*4.1.2. General procedure for the synthesis of 3-[2-Imino-2-(propylamino)ethyl]thymidine (**3a**), 3-[2-Imino-2-(butylamino)ethyl]thymidine (**3b**), 3-[2-Imino-2-(phenylamino)ethyl]thymidine (**3c**), and 3-[2-Imino-2-(benzylamino)ethyl]thymidine (**3d**).* Compound **2** (280 mg, 1 mmol) and amine (1.1 mmol) were dissolved in hexafluoroisopropanol (1 mL) in a closed pressure vessel (Quark Glass).[22] The reaction

mixture was heated at 95 ^{o}C overnight, suspended in water (80 mL), and extracted with ethyl acetate (50 mL × 3). The aqueous layer was evaporated under reduced pressure and the residue purified by preparative HPLC. The percentage purities of the synthesized compounds were > 95%.

*4.1.2.1. 3-[2-Imino-2-(propylamino)ethyl]thymidine (**3a**).* Retention time (analytical HPLC) = 9.1 min, yield = 80 mg, 24% (white amorphous solid); ^{1}H NMR (400 MHz, CD_3OD, δ ppm): 7.97 (s, 1H, H-6), 6.31 (t, *J* = 6.6 Hz, 1H, 1'H), 4.87 (s, 2H, N3-CH_2), 4.38-4.48 (m, 1H, 3'H), 3.95 (dd, *J* = 3.2 and 6.4 Hz, 1H, 4'H), 3.82 (dd, *J* = 3.0 and 12.0 Hz, 1H, 5"H), 3.75 (dd, *J* = 3.5 and 12.0 Hz, 1H, 5'H), 3.25 (t, *J* = 7.2 Hz, 2H, =NH-CH_2), 2.18-2.35 (m, 2H, 2'H and 2"H), 1.94 (s, 3H, 5-CH_3), 1.60-1.71 (m, 2H, =NH-CH_2-CH_2), 0.99 (t, *J* = 7.4 Hz, 3H, propyl-CH_3). ^{13}C NMR (CD_3OD, 100 MHz, δ ppm): 165.18 (C-4 C=O), 165.08 (HN=*C*-NH), 152.35 (C-2 C=O), 137.57 (C-6), 110.77 (C-5), 89.18 (C-1'), 87.51 (C-4'), 72.28 (C-3'), 62.86 (C-5'), 45.33 (N3-*C*H_2), 42.50 (=NH-*C*H_2), 41.43 (C-2'), 22.04 (=NH-CH_2-*C*H_2), 13.15 (5-*C*H_3), 11.52 (propyl-*C*H_3); HR-MS (ESI-MS) for $C_{15}H_{25}N_4O_5$. Calcd: 341.1825. Found: *m/z* 341.1799 $(M+H)^+$.

*4.1.2.2. 3-[2-Imino-2-(butylamino)ethyl]thymidine (**3b**).* Retention time (analytical HPLC) = 10 min, yield = 160 mg, 45% (white amorphous solid); ^{1}H NMR (400 MHz, CD_3OD, δ ppm): 7.98 (s, 1H, H-6), 6.32 (t, *J* = 6.5 Hz, 1H, 1'H), 4.88 (s, 2H, N3-CH_2), 4.37-4.47 (m, 1H, 3'H), 3.90-4.00 (m, 1H, 4'H), 3.83 (dd, *J* = 2.5 and 11.9 Hz, 1H, 5"H), 3.76 (dd, *J* = 3.4 and 11.9 Hz, 1H, 5'H), 3.25-3.35 (m, 2H, Methanol-CH_3 and NH-CH_2), 2.20-2.38 (m, 2H, 2'H and 2"H), 1.96 (s, 3H, 5-CH_3), 1.58-1.72 (m, 2H, NH-CH_2-CH_2),

1.36-1.50 (m, 2H, butyl-C*H*$_2$-CH$_3$), 0.99 (t, *J* = 7.2 Hz, 3H, butyl-C*H*$_3$). ^{13}C NMR (CD_3OD, 100 MHz, δ ppm): 165.00 (C-4 C=O), 152.27 (C-2 C=O), 137.50 (C-6), 110.70 (C-5), 89.09 (C-1'), 87.44 (C-4'), 72.20 (C-3'), 62.79 (C-5'), 43.47 (N3-*C*H$_2$), 42.42 (NH-*C*H$_2$), 41.35 (C-2'), 30.63 (NH-CH$_2$-*C*H$_2$), 20.95 (butyl-*C*H$_2$-*C*H$_3$), 13.98 (butyl-*C*H$_3$), 13.08 (5-*C*H$_3$); HR-MS (ESI-MS) for $C_{16}H_{27}N_4O_5$. Calcd: 355.1981. Found: *m/z* 355.1985 $(M+H)^+$.

*4.1.2.3. 3-[2-Imino-2-(phenylamino)ethyl]thymidine (**3c**).* Retention time (analytical HPLC) = 7.6 min, yield = 290 mg, 79% (white amorphous solid); ^{1}H NMR (400 MHz, CD_3OD, δ ppm): 8.00 (s, 1H, H-6), 7.45-7.60 (m, 3H, phenyl-Ar-H), 7.34 (d, *J* = 7.4 Hz, 2H, phenyl-Ar-H), 6.35 (t, *J* = 6.5 Hz, 1H, 1'H), 5.07 (dd, *J* = 16.5 and 23.4 Hz, 2H, N3-C*H*$_2$), 4.38-4.48 (m, 1H, 3'H), 3.95 (dd, *J* = 3.2 and 6.4 Hz, 1H, 4'H), 3.83 (dd, *J* = 3.0 and 12.0 Hz, 1H, 5''H), 3.76 (dd, *J* = 3.4 and 12.0 Hz, 1H, 5'H), 2.20-2.38 (m, 2H, 2'H and 2''H), 1.98 (s, 3H, 5-C*H*$_3$). ^{13}C NMR (CD_3OD, 100 MHz, δ ppm): 166.13 (C-4 C=O), 165.09 (HN=*C*-NH), 152.35 (C-2 C=O), 137.61 (phenyl-Ar-*C*), 135.04 (C-6), 131.56, 130.49, 126.83 (phenyl-Ar-*C*), 110.75 (C-5), 89.14 (C-1'), 87.52 (C-4'), 72.21 (C-3'), 62.80 (C-5'), 42.66 (N3-*C*H$_2$), 41.38 (C-2'), 13.08 (5-*C*H$_3$); HR-MS (ESI-MS) for $C_{18}H_{23}N_4O_5$. Calcd: 375.1668. Found:. *m/z* 375.1677 $(M+H)^+$.

*4.1.2.4. 3-[2-Imino-2-(benzylamino)ethyl]thymidine (**3d**).* Retention time (analytical HPLC) = 11.8 min, yield = 310 mg, 81% (white amorphous solid); ^{1}H NMR (400 MHz, CD_3OD, δ ppm): 7.92 (s, 1H, H-6), 7.20-7.40 (m, 5H, benzyl-Ar-H), 6.31 (t, *J* = 6.5 Hz, 1H, 1'H), 4.65 (s, 2H, N3-C*H*$_2$), 4.42 (s, 3H, 3'H and NH-C*H*$_2$), 3.90-3.97 (m, 1H, 4'H),

3.83 (dd, *J* = 3.0 and 12.0 Hz, 1H, 5''H), 3.75 (dd, *J* = 3.4 and 12.0 Hz, 1H, 5'H), 2.20-2.38 (m, 2H, 2'H and 2''H), 1.95 (s, 3H, 5-CH_3). ^{13}C NMR (CD_3OD, 100 MHz, δ ppm): 168.79 (HN=*C*-NH), 164.19 (C-4 C=O), 151.30 (C-2 C=O), 138.66 (benzyl-Ar-*C*), 135.90 (C-6), 128.53, 127.45, 127.20 (benzyl-Ar-*C*), 109.53 (C-5), 87.87 (C-1'), 86.28 (C-4'), 71.02 (C-3'), 61.73 (C-5'), 43.35 (N3-*C*H_2), 43.12 (benzyl-*C*H_2), 40.38 (C-2'), 12.22 (5-*C*H_3); HR-MS (ESI-MS) for $C_{19}H_{25}N_4O_5$. Calcd: 389.1825. Found: *m/z* 389.1808 $(M+H)^+$.

*4.1.3. General procedure for the synthesis of 3-[2-Imino-2-{2-(closo-1,7-carboranyl)ethylamino}ethyl]thymidine (**3e**) and 3-[2-Imino-2-{2-(closo-1,7-carboranyl)propylamino}ethyl]thymidine (**3f**).* Carboranyl alkyl amine (1 mmol) and **2** (280 mg, 1 mmol) were dissolved in hexafluoroisopropanol (1 mL) in a round bottom flask and heated at 120 ^{o}C under argon atmosphere overnight to allow the solvent to evaporate. The reaction mixture was suspended in water (80 mL) and extracted with ethyl acetate (50 mL × 3). The aqueous layer was concentrated under reduced pressure and the residue purified by preparative HPLC. The percentage purities of the synthesized compounds were > 95%.

*4.1.3.1. 3-[2-Imino-2-{2-(closo-1,7-carboranyl)ethylamino}ethyl]thymidine (**3e**).* Retention time = (analytical HPLC) 14.8 min, yield = 32 mg, 7% (white wax); ^{1}H NMR (400 MHz, CD_3OD, δ ppm): 7.96 (s, 1H, H-6), 6.30 (t, *J* = 6.6 Hz, 1H, 1'H), 4.84 (s, 2H, N3-CH_2), 4.37-4.45 (m, 1H, 3'H), 3.90-3.96 (m, 1H, 4'H), 3.81 (dd, *J* = 2.9 and 12.0 Hz, 1H, 5''H), 3.75 (dd, *J* = 3.3 and 12.0 Hz, 1H, 5'H), 3.59 (s, 1H, Carboranyl *H*), 3.28-3.38

(m, 2H, C*D*$_3$-OD and NH-C*H*$_2$), 2.35 (t, *J* = 7.7 Hz, 2H, Carborane-C*H*$_2$-), 2.20-2.30 (m, 2H, 2'H and 2''H), 1.94 (s, 3H, 5-C*H*$_3$). ^{13}C NMR (CD_3OD, 100 MHz, δ ppm): 165.47 (C-4 C=O), 165.05 (HN=*C*-NH), 152.33 (C-2 C=O), 137.61 (C-6), 110.83 (C-5), 89.18 (C-1'), 87.52 (C-4'), 74.00 (Carboranyl *C*), 72.29 (C-3'), 62.88 (C-5'), 57.50 (Carboranyl *C*H), 43.07 (N3-*C*H$_2$), 42.60 (NH-*C*H$_2$), 41.42 (C-2'), 34.98 (Carborane-*C*H$_2$-), 13.17 (5-*C*H$_3$); HR-MS (ESI-MS) for $C_{16}H_{33}B_{10}N_4O_5$. Calcd: 469.3454. Found: *m/z* 469.3476 $(M+H)^+$.

*4.1.3.2. 3-[2-Imino-2-{3-(closo-1,7-carboranyl)propylamino}ethyl]thymidine (**3f**).* Retention time = (analytical HPLC) 15.2 min, yield = 48 mg, 10% (white wax); ^{1}H NMR (400 MHz, CD_3OD, δ ppm): 7.98 (s, 1H, H-6), 6.34 (t, *J* = 6.6 Hz, 1H, 1'H), 4.88 (s, 2H, N3-C*H*$_2$), 4.44-4.52 (m, 1H, 3'H), 3.94-4.00 (m, 1H, 4'H), 3.84 (dd, *J* = 3.0 and 12.0 Hz, 1H, 5''H), 3.77 (dd, *J* = 3.4 and 11.9 Hz, 1H, 5'H), 3.55 (s, 1H, Carboranyl *H*), 3.25 (t, *J* = 6.7 Hz, 2H, NH-C*H*$_2$), 2.22-2.36 (m, 2H, 2'H and 2''H), 2.04-2.16 (m, 2H, Carborane-C*H*$_2$-), 1.97 (s, 3H, 5-C*H*$_3$), 1.66-1.78 (m, 2H, Carborane-CH$_2$-C*H*$_2$-). ^{13}C NMR (CD_3OD, 100 MHz, δ ppm): 164.32 (C-4 C=O), 163.88 (HN=*C*-NH), 151.20 (C-2 C=O), 136.44 (C-6), 109.69 (C-5), 88.03 (C-1'), 86.35 (C-4'), 72.26 (Carboranyl *C*), 71.13 (C-3'), 61.73 (C-5'), 56.031 (Carboranyl *C*H), 48.86 (NH-*C*H$_2$-), 41.63 (N3-*C*H$_2$), 41.45 (NH-*C*H$_2$), 40.31 (C-2'), 33.70 (NH-CH$_2$-*C*H$_2$), 28.07 (Carborane-*C*H$_2$-), 12.05 (5-*C*H$_3$); HR-MS (ESI-MS) for $C_{17}H_{35}B_{10}N_4O_5$. Calcd: 483.3611. Found: *m/z* 483.3611 $(M+H)^+$.

*4.1.4. 3',5'-(Di-tert.-butyldimethylsilyl)-3-cyanothymidine (**5**).* 3',5'-(Di-*tert*.-butyldimethylsilyl)thymidine (**4**) [20] (10 g, 21.2 mmol) and cyanogen bromide (3 M solution in dichloromethane; 14 mL, 42 mmol) were dissolved in acetone (50 mL).

Triethylamine (2.75 mL, 20 mmol) was added at 0 ^{o}C and the reaction mixture was stirred for 2 h at this temperature [21]. Water (750 mL) was added and the resulting mixture was extracted with ethyl acetate (250 mL × 4). The organic layer was separated, dried using anhydrous magnesium sulfate (100 g), and evaporated. The residue was purified by silica gel column chromatography using hexanes and dichloromethane as solvent system. The compound was eluted at 50% dichloromethane in hexane. R_f: 0.63 (100% dichloromethane) yield = 8.5 g, 81% (white solid, m.p. 93 °C); ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 7.63 (s, 1H, H-6), 6.27 (q, *J* = 5.7 and 7.8 Hz, 1'H, 1H), 4.35-4.45 (m, 1H, 3'H), 3.96-4.06 (m, 1H, 4'H), 3.90 (dd, *J* = 2.4 and 11.5 Hz, 1H, 5''H), 3.78 (dd, *J* = 2.2 and 11.5 Hz, 1H, 5'H), 2.32 (ddd, *J* = 2.4, 5.7 and 13.1 Hz, 1H, 2''H), 1.99-2.10 (m, 1H, 2'H), 1.98 (s, 3H, 5-CH_3), 0.90 and 0.93 (two s, 18H, ≡C-CH_3), 0.08-0.14 (m, 12H, Si-CH_3). ^{13}C NMR ($CDCl_3$, 100 MHz, δ ppm): 159.82 (C-4 C=O), 147.82 (C-2 C=O), 136.51 (C-6), 109.81 (C-5), 102.75 (*C*≡N), 88.99 (C-1'), 86.98 (C-3'), 72.73 (C-4'), 63.42 (C-5'), 41.96 (C-2'), 26.32 and 26.11 (two ≡C-*C*H_3), 18.80 and 18.38 (two Si-*C*≡), 13.32 (5-*C*H_3), -4.44 and -4.99 (two Si-*C*H_3). IR: (N3-*CN*) 2264.9 cm^{-1}. HR-MS (ESI-MS) for $C_{23}H_{41}N_3NaO_5Si_2$. Calcd: 518.2482. Found: *m/z* 518.2462 $(M+Na)^+$.

*4.1.5. General procedure for the synthesis of 3-(Propylcarbamimidoyl)-3',5'-(di-tert.-butyldimethylsilyl)thymidine (**6a**), and 3-(Butylcarbamimidoyl)-3',5'-(di-tert.-butyldimethylsilyl)thymidine (**6b**)* [22]. Compound **5** (100 mg, 0.21 mmol) was dissolved in hexafluoroisopropanol (200 µL) at 0 °C. *n*-Propyl or *n*-butyl amine (0.4 mmol) was added and the reaction mixture was stirred for 5 h at 0 ^{o}C. The aqueous layer was evaporated under reduced pressure and the residue purified by preparative HPLC.

*4.1.5.1. 3-(Propylcarbamimidoyl)-3',5'-(di-tert.-butyldimethylsilyl)thymidine (**6a**).* R_f: 0.40 (100% dichloromethane), Retention time (preparative HPLC) = 46-52 min, yield = 111 mg, 95% (white amorphous solid); ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 7.47 (s, 1H, H-6), 6.28 (dd, *J* = 5.7 and 7.9 Hz, 1'H, 1H), 4.40-4.46 (m, 1H, 3'H), 3.91 (q, *J* = 2.3 Hz, 1H, 4'H), 3.84 (dd, *J* = 11.4 and 2.6 Hz, 1H, 5''H), 3.73 (dd, *J* = 11.4 and 2.3 Hz, 1H, 5'H), 3.16 (t, *J* = 7.0 Hz, 2H, NH-C*H*$_2$), 2.23 (ddd, J = 13.1, 5.7 and 2.5 Hz, 1H, 2''H), 1.93-2.03 (m, 1H, 2'H), 1.89 (s, 3H, 5-C*H*$_3$), 1.56-1.69 (m, 2H, NH-CH$_2$-C*H*$_2$), 0.95 (t, *J* = 7.4 Hz, 2H, propyl-C*H*$_3$), 0.85 and 0.90 (two s, 18H, ≡C-C*H*$_3$), 0.08 and 0.04 (two s, 12H , Si-C*H*$_3$). ^{13}C NMR ($CDCl_3$, 100 MHz, δ ppm): 162.82 (C-4 C=O), 150.02 (C-2 C=O), 135.58 (C-6), 111.35 (C-5), 88.87 (C-1'), 86.41 (C-3'), 73.18 (C-4'), 63.88 (C-5'), 46.61 (C-2'), 42.27 (NH-*C*H$_2$), 26.82 and 26.60 (≡C-*C*H$_3$), 23.26 (NH-CH$_2$-*C*H$_2$), 19.29 and 18.86 (two Si-*C*≡), 13.83 (5-*C*H$_3$), 12.49 (propyl-*C*H$_3$), -3.96 and -4.47 (two Si-*C*H$_3$). HR-MS (ESI-MS) for $C_{26}H_{51}N_4O_5Si_2$. Calcd: 555.3398. Found: *m/z* 555.3376 $(M+H)^+$.

*4.1.5.2. 3-(Butylcarbamimidoyl)-3',5'-(di-tert.-butyldimethylsilyl)thymidine (**6b**).* R_f: 0.45 (100% dichloromethane), Retention time (preparative HPLC) = 62-70 min, yield = 110 mg, 95% (white amorphous solid); ^{1}H NMR (400 MHz, CD_3OD, δ pp0m): 7.66 (s, 1H, H-6), 6.21 (dd, *J* = 5.8 and 8.0 Hz, 1'H, 1H), 4.40-4.54 (m, 1H, 3'H), 3.93 (dd, *J* = 3.1 and 5.8 Hz, 1H, 4'H), 3.68-3.90 (m, 2H, 5''H and 5'H), 3.42 (t, *J* = 7.0 Hz, 2H, NH-C*H*$_2$), 2.10-2.58 (m, 2H, 2''H and 2'H), 1.91 (s, 3H, 5-C*H*$_3$), 1.60-1.70 (m, 2H, NH-CH$_2$-C*H*$_2$-), 1.39-1.40 (m, 2H, butyl-C*H*$_2$-CH$_3$), 0.95 (t, *J* = 7.3 Hz, 2H, butyl-C*H*$_3$), 0.89 and 0.92 (two s, 18H, ≡C-C*H*$_3$), 0.09 and 0.11 (two s, 12H , Si-C*H*$_3$). ^{13}C NMR ($CDCl_3$, 100 MHz,

δ ppm): 162.48 (C-4 C=O), 153.79 (=N-*C*-NH), 149.68 (C-2 C=O), 137.66 (C-6), 111.12 (C-5), 88.57 (C-1'), 87.17 (C-3'), 73.77 (C-4'), 64.22 (C-5'), 44.21 (NH-*C*H_2), 41.72 (C-2'), 30.45(NH-CH_2-*C*H_2), 26.52 and 26.29 (≡C-*C*H_3), 20.81 (butyl-*C*H_2-CH_3), 19.32 and 18.88 (two Si-*C*≡), 13.91 (5-*C*H_3), 12.84 (butyl-*C*H_3), -4.59 and -5.18 (two Si-*C*H_3). HR-MS (ESI-MS) for $C_{27}H_{53}N_4O_5Si_2$. Calcd: 569.3554. Found: *m/z* 569.3551 $(M+H)^+$ and for $C_{27}H_{52}N_4NaO_5Si_2$. Calcd: 591.3374. Found: *m/z* 591.3370 $(M+Na)^+$.

*4.1.6. General procedure for the synthesis of 3-(Phenylcarbamimidoyl)-3',5'-(di-tert.-butyldimethylsilyl)thymidine (**6c**), and 3-(Benzylcarbamimidoyl)-3',5'-(di-tert.-butyldimethylsilyl)thymidine (**6d**)* [22]. Compound **5** (100 mg, 0.21 mmol) was dissolved in hexafluoroisopropanol (200 µL), aniline or benzyl amine (0.4 mmol) was added, and the reaction mixture was stirred for overnight at room temperature. The aqueous layer was evaporated under reduced pressure and the residue purified by preparative HPLC.

*4.1.6.1. 3-(Phenylcarbamimidoyl)-3',5'-(di-tert.-butyldimethylsilyl)thymidine (**6c**).* R_f: 0.70 (1% methanol in dichloromethane), Retention time (preparative HPLC) = 62-72 min, yield = 110 mg, 93% (white amorphous solid); ^{1}H NMR (400 MHz, CD_3OD, δ ppm): 7.61 (s, 1H, H-6), 7.20-7.40 (s, 2H, phenyl H), 6.90-7.20 (br s, 3H, phenyl H), 6.15-6.35 (m, 1H, 1'H), 4.48 (m, 1H, 3'H), 3.93-3.4.00 (m, 1H, 4'H), 3.90 (dd, *J* = 2.5 and 11.5 Hz, 1H, 5''H), 3.83 (dd, *J* = 1.8 and 11.5 Hz, 1H, 5'H), 2.10-2.30 (m, 2H, 2'H and 2''H), 1.92 (s, 3H, 5-C*H*$_3$), 0.96 and 0.95 (two s, 18H, ≡C-C*H*$_3$) 0.15 and 0.16 (two s, 12H, Si-C*H*$_3$). ^{13}C NMR (CD_3OD, 100 MHz, δ ppm): 163.69 (C-4 C=O), 150.69 (=N-*C*-NH), 147.64 (C-2 C=O), 136.55 (C-6), 130.30, 125.08, 123.14 (Aromatic-*C*), 111.16 (C-5), 89.36 (C-

1'), 86.94 (C-4'), 73.65 (C-3'), 64.15 (C-5'), 41.96 (C-2'), 26.55 and 26.35 (two ≡C-*C*H_3), 19.33 and 18.91 (two Si-*C*≡), 13.15 (5-*C*H_3), -4.40, -4.54, -5.14 (d, *J* = 9.8 Hz, Si-*C*H_3), -5.18 (d, *J* = 3.0 Hz, Si-*C*H_3); HR-MS (ESI-MS) for $C_{29}H_{49}N_4O_5Si_2$. Calcd: 589.3241. Found: *m/z* 589.3251 $(M+H)^+$.

*4.1.6.2. 3-(Benzylcarbamimidoyl)-3',5'-(di-tert.-butyldimethylsilyl)thymidine (**6d**).* R_f: 0.75 (1% methanol in dichloromethane), Retention time (preparative HPLC) = 47-52 min, yield = 120 mg, >99% (white amorphous solid); ^{1}H NMR (400 MHz, CD_3OD, δ ppm): 7.74 (d, *J* = 1.2 Hz, 1H, H-6), 7.35-7.53 (m, 5H, phenyl H), 6.29 (dd, *J* = 5.9 and 7.8 Hz, 1H, 1'H), 4.73 (s, 2H, NH-C*H*$_2$-), 4.49-4.53 (m, 1H, 3'H), 4.00 (dd, *J* = 3.2 and 5.8 Hz, 1H, 4'H), 3.92 (dd, *J* = 3.3 and 11.5 Hz, 1H, 5''H), 3.85 (dd, *J* = 3.2 and 11.5 Hz, 1H, 5'H), 2.29 (ddd, *J* = 2.7, 5.9 and 13.3 Hz, 1H, 2'H), 2.16-2.24 (m, 1H, 2''H), 1.84 (s, 3H, 5-C*H*$_3$), 0.96 and 0.98 (two s, 18H, ≡C-C*H*$_3$), 0.15 and 0.17 (two s, 12H, Si-C*H*$_3$). ^{13}C NMR (CD_3OD, 100 MHz, δ ppm): 162.58 (C-4 C=O), 154.19 (=N-*C*-NH), 149.78 (C-2 C=O), 137.80 (C-6), 134.77, 130.15, 129.64, 128.86 (Aromatic-*C*), 111.19 (C-5), 89.67 (C-1'), 87.26 (C-4'), 73.86 (C-3'), 64.28 (C-5'), 47.98 (NH-*C*H_2-), 41.78 (C-2'), 26.51 and 26.28 (two ≡C-*C*H_3), 19.34 and 18.90 (two Si-*C*≡), 12.84 (5-*C*H_3), -4.56 (d, *J* = 9.8 Hz, Si-*C*H_3), -5.18 (d, *J* = 3.0 Hz, Si-*C*H_3); HR-MS (ESI-MS) for $C_{30}H_{51}N_4O_5Si_2$. Calcd: 603.3398. Found: *m/z* 603.3331 $(M+H)^+$ and for $C_{30}H_{50}N_4NaO_5Si_2$. Calcd: 625.3217. Found: *m/z* 625.3253 $(M+Na)^+$.

*4.1.7. General procedure for the synthesis of 3-[2-(closo-1,7-Carboranyl)ethylcarbamimidoyl]-3',5'-(di-tert.-butyldimethylsilyl)thymidine (**6e**), 3-[3-*

*(closo-1,7-Carboranyl)propylcarbamimidoyl]-3',5'-(di-tert.-butyldimethylsilyl)thymidine (**6f**), and 3-[4-(closo-1,7-Carboranyl)butylcarbamimidoyl]-3',5'-(di-tert.-butyldimethylsilyl)thymidine (**6g**)* [22]. Compound **11** (100 mg, 0.21 mmol) was dissolved in hexafluoroisopropanol (200 µL) and *closo*-1,7-carboranyl)alkyl amine (0.4 mmol) was added. The reaction mixture was stirred overnight at 90 °C. The aqueous layer was evaporated under reduced pressure and the residue purified by preparative HPLC.

4.1.7.1. 3-[2-(closo-1,7-Carboranyl)ethylcarbamimidoyl]-3',5'-(di-tert.-butyldimethylsilyl)thymidine (6e). R_f: 0.85 (1% methanol in dichloromethane), Retention time (preparative HPLC) = 42-47 min, yield = 44 mg, >32% (white amorphous solid); ^{1}H NMR (400 MHz, CD_3OD, δ ppm): 7.70 (s, 1H, H-6), 6.24 (t, *J* = 6.6 Hz, 1'H, 1H), 4.45-4.55 (m, 1H, 3'H), 3.93-4.00 (m, 1H, 4'H), 3.89 (dd, *J* = 2.8 and 11.5 Hz, 1H, 5''H), 3.83 (dd, *J* = 2.5 and 11.5 Hz, 1H, 5'H), 3.60 (Carbranyl-*H*), 3.50 (t, *J* = 7.6 Hz, 2H, NH-C*H*$_2$), 2.40 (t, *J* = 7.6 Hz, 2H, Carborane-C*H*$_2$), 2.11-2.30 (m, 2H, 2'H and 2''H), 1.94 (s, 3H, 5-C*H*$_3$), 0.93 and 0.96 (two s, 18H, ≡C-C*H*$_3$), 0.13 and 0.15 (two s, 12H, Si-C*H*$_3$). ^{13}C NMR (CD_3OD, 100 MHz, δ ppm): 162.59 (C-4 C=O), 153.93 (=N-*C*-NH), 149.79 (C-2 C=O), 137.86 (C-6), 111.28 (C-5), 89.73 (C-1'), 87.34 (C-4'), 73.89 (Carboranyl-*C*/C-3'), 64.36 (C-5'), 57.54 (Carboranyl-*CH*), 43.96 (NH-*C*H$_2$), 41.85 (C-2'), 35.11 (Carborane-*C*H$_2$), 26.64 and 26.41 (two ≡C-*C*H$_3$), 19.45 and 19.01 (two Si-*C*≡), 12.95 (5-*C*H$_3$), -4.46 and -5.04 (two Si-*C*H$_3$); HR-MS (ESI-MS) for $C_{27}H_{59}B_{10}N_4O_5Si_2$. Calcd: 683.5027. Found: *m/z* 683.5076 $(M+H)^+$.

*4.1.7.2. 3-[3-(closo-1,7-Carboranyl)propylcarbamimidoyl]-3',5'-(di-tert.-butyldimethylsilyl)thymidine (**6f**).* The reaction conditions described above resulted in a mixture of 3-[3-(*closo*-1,7-carboranyl)propylcarbamimidoyl]thymidine (**7f**) along with 3′/5′-mono-TBDMS- and 3′,5′-di-TBDMS-3-[3-(*closo*-1,7-carboranyl)propylcarbamimidoyl] dThd (**6f**). Product formation was confirmed by MS using a small quantity of the reaction mixture. Deprotection of remaining TBDMS-groups was accomplished *in situ* by adding trifluoroacetic acid in dichloromethane (10 mL, 10%, v/v) to the reaction mixture for 4 h at room temperature to give **7f** in 29% overall yield. R_f: 0.85 (1% methanol in dichloromethane), HR-MS (ESI-MS) for **6f** ($C_{28}H_{61}B_{10}N_4O_5Si_2$). Calcd: 697.5184. Found: *m/z* 697.5167 $(M+H)^+$. HR-MS (ESI-MS) for 3′/5′-mono-TBDMS-3-[3-(*closo*-1,7-carboranyl)propylcarbamimidoyl]thymidine ($C_{22}H_{47}B_{10}N_4O_5Si$). Calcd: 583.4319. Found: *m/z* 583.3486 $(M+H)^+$.

*4.1.7.3. 3-[4-(closo-1,7-Carboranyl)butylcarbamimidoyl]-3',5'-(di-tert.-butyldimethylsilyl)thymidine (**6g**).* R_f: 0.90 (1% methanol in dichloromethane), Retention time (preparative HPLC) = 49-55 min, yield = 55 mg, >38% (white amorphous solid); ^{1}H NMR (400 MHz, CD_3OD, δ ppm): 7.72 (s, 1H, H-6), 6.25 (t, *J* = 6.6 Hz, 1'H, 1H), 4.45-4.55 (m, 1H, 3'H), 4.10 (q, *J* = 7.0 Hz, 1H, 4'H), 3.90 (dd, *J* = 2.5 and 11.5 Hz, 1H, 5"H), 3.84 (dd, *J* = 2.0 and 11.5 Hz, 1H, 5'H), 3.40-3.60 (m, 3H, Carbranyl-*H* and NH-C*H*$_2$), 2.96-3.20 (m, 2H, Carborane-C*H*$_2$), 2.10-2.30 (m, 2H, 2'H and 2"H) 1.96-2.10 (m, 2H, NH-CH$_2$C*H*$_2$), 1.96 (s, 3H, 5-C*H*$_3$), 1.40-1.60 (m, 2H, Carborane-CH$_2$C*H*$_2$), 0.93 and 0.96 (two s, 18H, ≡C-C*H*$_3$), 0.13 and 0.15 (two s, 12H, Si-C*H*$_3$). ^{13}C NMR (CD_3OD, 100 MHz, δ ppm): 162.53 (C-4 C=O), 154.09 (=N-*C*-NH), 149.78 (C-2 C=O), 137.84 (C-6),

111.26 (C-5), 89.80 (C-1'), 87.37 (C-4'), 77.59 (Carboranyl-*C*), 74.03 (C-3'), 64.42 (C-5'), 57.02 (Carboranyl-*CH*), 46.23 (NH-*C*H_2), 41.88 (C-2'), 37.71 (Carborane-*C*H_2), 30.51 (NH-CH_2-*C*H_2), 28.25 (Carborane-CH_2-*C*H_2), 28.38 and 28.61 (two ≡C-*C*H_3), 19.44 and 19.00 (two Si-*C*≡), 12.93 (5-*C*H_3), -4.38 and -5.05 (two Si-*C*H_3); HR-MS (ESI-MS) for $C_{29}H_{63}B_{10}N_4O_5Si_2$. Calcd: 711.5340. Found: *m/z* 711.5376 $(M+H)^+$.

*4.1.8. General procedure for the synthesis of 3-(Carbamimidoyl)thymidine derivatives (**7a**-**7g**).* Compounds **6a**-**6g** (40 mg) were added to a solution of TFA in dichloromethane (10 mL, 10% v/v). The reaction mixtures were stirred at room temperature for 4 h, the solvents were evaporated under reduced pressure, and the residues were purified by preparative HPLC. The percentage purity of the synthesized compounds were > 95%.

*4.1.8.1. 3-(Propylcarbamimidoyl)thymidine (**7a**).* Retention time (analytical HPLC) = 4.8 min, yield = 9 mg, 40% (white amorphous solid); ^{1}H NMR (400 MHz, $CDOD_3$, δ ppm): 8.08 (s, 1H, H-6), 6.28 (t, *J* = 6.3 Hz, 1'H, 1H), 4.40-4.46 (m, 1H, 3'H), 3.96 (q, *J* = 3.0 Hz, 1H, 4'H), 3.82 (dd, *J* = 12.0 and 3.0 Hz, 1H, 5''H), 3.76 (dd, *J* = 12.0 and 3.3 Hz, 1H, 5'H), 3.42 (t, *J* = 6.3 Hz, 2H, NH-C*H*$_2$), 2.20-2.35 (m, 2H, 2''H and 2'H), 1.94 (s, 3H, 5-C*H*$_3$), 1.69-1.79 (m, 2H, NH-CH_2-C*H*$_2$), 1.06 (t, *J* = 7.4 Hz, 2H, propyl-C*H*$_3$). ^{13}C NMR ($CDOD_3$, 100 MHz, δ ppm): 162.66 (C-4 C=O), 154.02 (NH-*C*(=NH)-), 149.81 (C-2 C=O), 138.65 (C-6), 110.98 (C-5), 89.40 (C-1'), 87.29 (C-3'), 72.25 (C-4'), 62.73 (C-5'), 46.11 (C-2'), 41.43 (NH-*C*H_2), 21.95 (NH-CH_2-*C*H_2), 12.59 (5-*C*H_3), 11.44 (propyl-*C*H_3). HR-MS (ESI-MS) for $C_{14}H_{23}N_4O_5$. Calcd: 327.1668. Found: *m/z* 327.1654 $(M+H)^+$.

*4.1.8.2. 3-(Butylcarbamimidoyl)thymidine (**7b**).* Retention time (analytical HPLC) = 4.4 min, yield = 11 mg, 46% (white amorphous solid); ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 8.07 (s, 1H, H-6), 6.28 (t, *J* = 6.6 Hz, 1H, 1'H), 4.40-4.60 (m, 1H, 3'H), 3.90-4.00 (m, 1H, 4'H), 3.83 (dd, *J* = 3.0, 12.0 Hz, 1H, 5"H), 3.76 (dd, *J* = 3.3, 12.0 Hz, 1H, 5'H), 3.46 (t, *J* = 6.9 Hz, 2H, NH-C*H*$_2$-), 2.18-2.38 (m, 2H, 2"H and 2'H), 1.94 (s, 3H, 5-C*H*$_3$), 1.65-1.75 (m, 2H, NH-CH$_2$-C*H*$_2$-), 1.46-155 (m, 2H, butyl-C*H*$_2$-CH$_3$), 0.99 (t, *J* = 7.3 Hz, 3H, butyl-C*H*$_3$). ^{13}C NMR ($CDCl_3$, 100 MHz, δ ppm): 162.69 (C-4 C=O), 153.85 (NH-*C*(=NH)-), 149.77 (C-2 C=O), 138.58 (C-6), 110.95 (C-5), 89.26 (C-1'), 87.26 (C-3'), 72.15 (C-4'), 62.67 (C-5'), 44.20 (NH-*C*H$_2$), 41.34 (C-2'), 30.44 (NH-CH$_2$-*C*H$_2$), 20.79 (butyl-*C*H$_2$-CH$_3$), 13.87 (5-*C*H$_3$), 12.60 (butyl-*C*H$_3$). HR-MS (ESI-MS) for $C_{15}H_{25}N_4O_5$. Calcd: 341.1825. Found: *m/z* 341.1853 $(M+H)^+$.

*4.1.8.3. 3-(Phenylcarbamimidoyl)thymidine (**7c**).* Retention time (analytical HPLC) = 8.9 min, yield = 18 mg, 70% (white amorphous solid); ^{1}H NMR (400 MHz, CD_3OD, δ ppm): 8.11 (s, 1H, H-6), 7.35-7.63 (m, 5H, phenyl H), 6.27-6.40 (m, 1'H, 1H), 4.35-4.50 (m, 1H, 3'H), 3.90-4.00 (m, 1H, 4'H), 3.84 (d, *J* = 11.1 Hz, 1H, 5"H), 3.77 (d, *J* = 11.4 Hz, 1H, 5'H), 2.20-2.40 (m, 2H, 2'H and 2"H), 1.90-2.05 (s, 3H, 5-C*H*$_3$). ^{13}C NMR (CD_3OD, 100 MHz, δ ppm): 162.84 (C-4 C=O), 153.50 (=N-*C*-NH), 149.96 (C-2 C=O), 138.68 (C-6), 135.96, 131.59, 130.20, 135.53 (Ar-*C*), 111.04 (C-5), 89.38 (C-1'), 87.33 (C-4'), 72.20 (C-3'), 62.71 (C-5'), 41.47 (C-2'), 12.63 (5-*C*H$_3$); HR-MS (ESI-MS) for $C_{17}H_{21}N_4O_5$. Calcd: 361.1512. Found: *m/z* 361.1532 $(M+H)^+$.

*4.1.8.4. 3-(Benzylcarbamimidoyl)thymidine (**7d**).* Retention time (analytical HPLC) = 9.6 min, yield = 20 mg, 80% (white amorphous solid); ^{1}H NMR (400 MHz, CD_3OD, δ ppm): 8.08 (s, 1H, H-6), 7.34-7.50 (m, 5H, phenyl H), 6.29 (t, 6.6 Hz, 1H, 1'H), 4.71 (s, 2H, NH-C*H*$_2$-), 4.40-4.47 (m, 1H, 3'H), 3.96 (dd, *J* = 2.9 and 5.9 Hz, 1H, 4'H), 3.83 (dd, *J* = 2.9 and 12.0 Hz, 1H, 5"H), 3.76 (dd, *J* = 3.3 and 12.0 Hz, 1H, 5'H), 2.21-2.37 (m, 2H, 2'H and 2"H), 1.96 (s, 3H, 5-C*H*$_3$). ^{13}C NMR (CD_3OD, 100 MHz, δ ppm): 162.28 (C-4 C=O), 154.27 (=N-*C*-NH), 149.87 (C-2 C=O), 138.69 (C-6), 134.78, 130.13, 129.63, 128.86 (Aromatic-*C*), 111.00 (C-5), 89.35 (C-1'), 87.33 (C-4'), 72.18 (C-3'), 62.69 (C-5'), 47.97 (NH-*C*H$_2$-), 41.42 (C-2'), 12.60 (5-*C*H$_3$); HR-MS (ESI-MS) for $C_{18}H_{23}N_4O_5$. Calcd: 375.1668. Found: 375.1673 $(M+H)^+$.

*4.1.8.5. 3-[2-(closo-1,7-Carboranyl)ethylcarbamimidoyl]thymidine (**7e**).* Retention time (analytical HPLC) = 15.0 min, yield = 18 mg, 69% (white wax); ^{1}H NMR (400 MHz, CD_3OD, δ ppm): 8.08 (s, 1H, H-6), 6.27 (t, *J* = 6.6 Hz, 1'H, 1H), 4.45-4.50 (m, 1H, 3'H), 3.93-4.00 (m, 1H, 4'H), 3.82 (dd, *J* = 2.8 and 12.0 Hz, 1H, 5"H), 3.75 (dd, *J* = 3.0 and 12.0 Hz, 1H, 5'H), 3.62 (Carboranyl-C*H*), 3.51 (t, *J* = 7.6 Hz, 2H, NH-C*H*$_2$), 2.40 (t, *J* = 7.6 Hz, 2H, Carborane-C*H*$_2$), 2.2-2.35 (m, 2H, 2'H and 2"H), 1.94 (s, 3H, 5-C*H*$_3$). ^{13}C NMR (CD_3OD, 100 MHz, δ ppm): 162.67 (C-4 C=O), 154.29 (=N-*C*-NH), 149.81 (C-2 C=O), 138.84 (C-6), 111.07 (C-5), 89.52 (C-1'), 87.42 (C-4'), 73.84 (Carboranyl-*C*), 72.34 (C-3'), 62.81 (C-5'), 57.57 (Carboranyl-*CH*), 43.82 (NH-*C*H$_2$), 41.51 (C-2'), 34.99 (Carborane-*C*H$_2$), 12.65 (5-*C*H$_3$); HR-MS (ESI-MS) for $C_{15}H_{31}B_{10}N_4O_5$. Calcd: 455.3298. Found: *m/z* 455.3270 $(M+H)^+$.

*4.1.8.6. 3-[3-(closo-1,7-Carboranyl)propylcarbamimidoyl]thymidine (**7f**).* Retention time (analytical HPLC) = 20.2 min, yield = 22 mg, 29% (white wax); ^{1}H NMR (400 MHz, CD_3OD, δ ppm): 8.08 (s, 1H, H-6), 6.29 (t, *J* = 6.7 Hz, 1'H, 1H), 4.40-4.45 (m, 1H, 3'H), 3.90-3.95 (m, 1H, 4'H), 3.82 (dd, *J* = 2.9 and 12.0 Hz, 1H, 5''H), 3.75 (dd, *J* = 3.2 and 12.0 Hz, 1H, 5'H), 3.54 (s, 1H, Carboranyl-C*H*), 3.41 (t, *J* = 6.5 Hz, 2H, NH-CH_2), 2.10-2.32 (m, 4H, 2'H and 2''H, Carborane-CH_2), 1.95 (s, 3H, 5-CH_3), 1.74-1.83 (m, 2H, NH-CH_2-CH_2). ^{13}C NMR (CD_3OD, 100 MHz, δ ppm): 162.66 (C-4 C=O), 153.98 (=N-*C*-NH), 149.85 (C-2 C=O), 138.65 (C-6), 111.02 (C-5), 89.43 (C-1'), 87.30 (C-4'), 76.67 (Carboranyl-*C*), 72.30 (C-3'), 62.76 (C-5'), 57.11 (Carboranyl-*C*H), 43.54 (NH-*C*H_2), 41.42 (C-2'), 34.68 (Carborane-*C*H_2), 29.10 (Carborane-CH_2-*C*H_2), 12.61 (5-*C*H_3); HR-MS (ESI-MS) for $C_{16}H_{33}B_{10}N_4O_5$. Calcd: 469.3454. Found: *m/z* 469.3382 $(M+H)^+$.

*4.1.8.7. 3-[4-(closo-1,7-Carboranyl)butylcarbamimidoyl]thymidine (**7g**).* Retention time (analytical HPLC) = 21.2 min, yield = 21 mg, 80% (white wax); ^{1}H NMR (400 MHz, CD_3OD, δ ppm): 8.08 (s, 1H, H-6), 6.29 (t, *J* = 6.7 Hz, 1'H, 1H), 4.40-4.44 (m, 1H, 3'H), 3.92-4.00 (m, 1H, 4'H), 3.83 (dd, *J* = 2.7 and 12.0 Hz, 1H, 5''H), 3.76 (dd, *J* = 2.9 and 12.0 Hz, 1H, 5'H), 3.51 (s, 1H, Carboranyl-C*H*), 3.44 (t, *J* = 6.4 Hz, 2H, NH-CH_2), 2.10-2.32 (m, 2H, 2'H and 2''H), 2.05 (t, *J* = 8.2 Hz, 2H, Carborane-CH_2), 1.95 (s, 3H, 5-CH_3), 1.40-1.70 (m, 4H, NH-CH_2CH_2CH_2). ^{13}C NMR (CD_3OD, 100 MHz, δ ppm): 162.66 (C-4 C=O), 159.45 (=N-*C*-NH), 154.17 (C-2 C=O), 138.74 (C-6), 111.06 (C-5), 89.49 (C-1'), 87.40 (C-4'), 77.58 (Carboranyl-*C*), 72.34 (C-3'), 62.82 (C-5'), 57.03 (Carboranyl-*C*H), 44.06 (NH-*C*H_2-), 41.51 (C-2'), 37.59 (Carborane-*C*H_2), 28.25 and 28.13 (NH-CH_2-*C*H_2-

CH_2), 12.66 (5-*C*H_3); HR-MS (ESI-MS) for $C_{17}H_{35}B_{10}N_4O_5$. Calcd: 483.3611. Found: *m/z* 483.3558 $(M+H)^+$.

*4.1.9. 3-(Tetrazol-5-ylmethyl)thymidine (**8**).* A mixture of **2** (500 mg, 1.75 mmol), sodium azide (350 mg, 5.4 mmol), and zinc bromide (350 mg, 1.5 mmol) were dissolved in water/isopropanol (15 mL, 2/1, v/v) and heated in a sealed tube at 90 °C for three days [23]. The reaction mixture was passed through a 0.2 µm filter (Fisher Scientific, USA), the filtrate evaporated under reduced pressure, and the residue purified by preparative HPLC. Retention time (analytical HPLC) = 15.5 min, Yield = 575 mg, 90% (white amorphous solid); ^{1}H NMR (400 MHz, CD_3OD, δ ppm): 7.92 (s, 1H, H-6), 6.31 (t, *J* = 6.6 Hz, 1H, 1'H), 5.44 (s, 2H, N3-C*H*$_2$), 4.38-4.48 (m, 1H, 3'H), 3.90-4.00 (m, 1H, 4'H), 3.83 (dd, *J* = 3.0 and 12.0 Hz, 1H, 5''H), 3.76 (dd, *J* = 3.6 and 12.0 Hz, 1H, 5'H), 2.20-2.38 (m, 2H, 2'H and 2''H), 1.92 (s, 3H, 5-C*H*$_3$). ^{13}C NMR (CD_3OD, 100 MHz, δ ppm): 164.72 (C-4 C=O), 154.93 (=N-*C*=N-), 152.00 (C-2 C=O), 137.09 (C-6), 110.68 (C-5), 88.82 (C-1'), 87.23 (C-4'), 72.00 (C-3'), 62.70 (C-5'), 41.23 (C-2'), 35.62 (N3-*C*H_2), 13.13 (5-*C*H_3); HR-MS (ESI-MS) for $C_{12}H_{17}N_6O_5$. Calcd: 325.1260. Found: *m/z* 325.1275 $(M+H)^+$, for $C_{12}H_{16}N_6NaO_5$. Calcd: 347.1080. Found: *m/z* 347.1108 $(M+Na)^+$ and for $C_{24}H_{33}N_{12}O_{10}$. Calcd: 649.2443. Found: *m/z* 649.2494 $(2M+H)^+$.

*4.1.10. 3-(Tetrazol-5-yl)thymidine (**10**).* A mixture of **2** (100 mg, 0.36 mmol), sodium azide (70 mg, 1.1 mmol), and zinc bromide (70 mg, 0.3 mmol) were dissolved in a mixture of DMF, water, and isopropanol (6 mL, 3/2/1, v/v/v). The reaction mixture was stirred for 5 h at 0 ^{o}C, then suspended in water (200 mL), and extracted with ethyl acetate

(100 mL × 3) [23]. The organic layer was evaporated, the residue dissolved in TFA in dichloromethane (10 mL, 10% v/v), and the resulting mixture was stirred for 4 h at room temperature. The solvents were evaporated and the residue was purified by preparative HPLC. Retention time (analytical HPLC) = 13.2 min, Yield = 25 mg, 44% (white amorphous solid); ^{1}H NMR (400 MHz, CD_3OD, δ ppm): 8.05 (s, 1H, H-6), 6.28 (t, *J* = 6.5 Hz, 1H, 1'H), 4.43 (dd, *J* = 4.8 and 8.2 Hz, 1H, 3'H), 3.94 (dd, *J* = 3.2 and 6.5 Hz, 1H, 4'H), 3.84 (dd, *J* = 3.0 and 12.1 Hz, 1H, 5''H), 3.76 (dd, *J* = 3.5 and 12.0 Hz, 1H, 5'H), 2.30 (t, *J* = 5.4 Hz, 2H, 2'H and 2''H), 1.96 (m, 3H, 5-C*H*$_3$). ^{13}C NMR (CD_3OD, 100 MHz, δ ppm): 165.06 (N-*C*=N), 164.67 (C-4 C=O), 151.51 (C-2 C=O), 138.43 (C-6), 111.12 (C-5), 89.23 (C-1'), 87.55 (C-4'), 72.11 (C-3'), 62.80 (C-5'), 41.54 (C-2'), 13.03 (5-*C*H$_3$); HR-MS (ESI-MS) for $C_{11}H_{14}N_6NaO_5$. Calcd: 333.0923. Found: *m/z* 333.0939 $(M+Na)^+$.

*4.1.11. General procedure for the synthesis of N-alkylated tetrazolyl-type thymidine derivatives (**9a**, **9b1/2-9d1/2** and **11a**, **11b1/2-11d1/2**). Compound* **8** or **10** (0.046 mmol), iodoalkyl derivative (0.09 mmol), and potassium carbonate (20 mg, 0.15 mmol) were dissolved in DMF, and acetone (2 mL, 1/1, v/v) and stirred at 50 °C for 24 h. The residues were purified as two isomers (N^b- and N^c-subsituted tetrazolyl dThd analogues) by preparative HPLC. The percentage purities of the synthesized compounds were > 95%.

*4.1.11.1. 3-[1(2)-Propyltetrazol-5-ylmethyl]thymidine (**9a**).* Retention time (analytical HPLC) = 22 min, yield = 10 mg, 59% (white amorphous solid). ^{1}H NMR (400 MHz, CD_3OD, δ ppm): 7.93 (s, 1H, H-6), 6.30 (t, *J* = 6.6 Hz, 1H, 1'H), 5.36 (s, 2H, N3-C*H*$_2$), 4.57 (t, *J* = 6.9 Hz, 2H, N^c-C*H*$_2$), 4.35-4.45 (m, 1H, 3'H), 3.87-3.95 (m, 1H, 4'H), 3.81 (dd,

J = 2.8 and 12.1 Hz, 1H, 5''H), 3.76 (dd, *J* = 3.5 and 12.1 Hz, 1H, 5'H), 2.18-2.38 (m, 2H, 2'H and 2''H), 2.00 (hexaplet, *J* = 7.2 Hz, 2H, N^c-CH_2-CH_2), 1.93 (s, 3H, 5-CH_3), 0.91 (t, *J* = 7.4 Hz, 2H, propyl-CH_3). ^{13}C NMR (CD_3OD, 100 MHz, δ ppm): 165.05 (C-4 C=O), 163.88 (=N-*C*=N-), 152.26 (C-2 C=O), 137.14 (C-6), 110.83 (C-5), 89.04 (C-1'), 87.40 (C-4'), 72.17 (C-3'), 62.88 (C-5'), 56.02 (N-*C*H_2), 41.23 (C-2'), 37.41 (N3-*C*H_2), 23.88 (N-CH_2-*C*H_2), 13.35 (5-*C*H_3), 11.33 (propyl-*C*H_3); HR-MS (ESI-MS) for $C_{15}H_{22}N_6NaO_5$. Calcd: 389.1549. Found: *m/z* 389.1536 $(M+Na)^+$.

*4.1.11.2. 3-[1(2)-closo-1,7-Carboranylethyltetrazol-5-ylmethyl]thymidine (**9b1/2**).*

Retention time (analytical HPLC) = 16.1 min, yield = 14 mg, 64% (white wax); isomeric ratio **9b1:9b2** = 20:80. ^{1}H NMR (400 MHz, CD_3OD, δ ppm): 7.93 (s, 1H, H-6), 6.29 (t, *J* = 6.6 Hz, 1H, 1'H), 5.35 (s, 2H, N3-CH_2), 4.65 (t, *J* = 7.5 Hz, 1.7 H, N^c-CH_2), 4.52 (t, *J* = 8.4 Hz, 0.3 H, N^b-CH_2), 4.35-4.45 (m, 1H, 3'H), 3.87-3.95 (m, 1H, 4'H), 3.81 (dd, *J* = 2.9 and 12.0 Hz, 1H, 5''H), 3.74 (dd, *J* = 3.5 and 12.0 Hz, 1H, 5'H), 3.55 (s, 1H, Carboranyl *H*), 2.75 (t, *J* = 7.5 Hz, 2H, Carborane-CH_2), 2.19-2.33 (m, 2H, 2'H and 2''H), 1.93 (s, 3H, 5-CH_3). ^{13}C NMR (CD_3OD, 100 MHz, δ ppm): 164.96 (C-4 C=O), 164.07 (=N-*C*=N-), 152.19 (C-2 C=O), 137.12 (C-6), 110.80 (C-5), 89.06 (C-1'), 87.35 (C-4'), 73.64 (Carboranyl *C*), 72.17 (C-3'), 62.84 (C-5'), 57.54 (Carboranyl *C*H), 52.99 (N-*C*H_2), 41.53 (C-2'), 37.28 (Carborane-*C*H_2), 36.07 (N3-*C*H_2), 13.30 (5-*C*H_3); HR-MS (ESI-MS) for $C_{16}H_{31}B_{10}N_6O_5$. Calcd: 495.3359. Found: *m/z* 495.3372 $(M+H)^+$ and for $C_{16}H_{30}B_{10}N_6NaO_5$. Calcd: 517.3179. Found: *m/z* 517.3160 $(M+Na)^+$.

^{1}H-^{1}H NOESY (800 MHz, CD_3OD): Cross-peak observed between the triplet at δ 4.52 and the singlet at 5.35 ppm. T1 relaxation rates were measured using an inversion

recovery experiment at 250 MHz and were calculated using TopSpin 3.1 (Bruker, USA). A ^{1}H-^{1}H NOESY with presaturation on the methanol-OH at δ 4.892 ppm was collected at 800 MHz in CD_3OD with a NOESY mixing time of 0.69 s based on the average T1 relaxation time of the protons of interest, a spectral width of 1.7 ppm in both dimensions centered at 4.874 ppm, 256 points in the indirect dimension, 1024 points in the direct dimension, and a 3 s recycle delay. The NOESY data were processed using TopSpin 3.1. The data were zero filled to 2048 points in the direct dimension and 1024 points in the indirect dimension (see Supplementary material).

*4.1.11.3. 3-[1(2)-closo-1,7-Carboranylpropyltetrazol-5-ylmethyl]thymidine (**9c1/2**).* Retention time (analytical HPLC) = 16.8 min, yield = 14 mg, 60% (white wax); isomeric ratio **9c1:9c2** = 15:85. ^{1}H NMR (400 MHz, CD_3OD, δ ppm): 7.95 (s, 1H, H-6), 6.31 (t, *J* = 6.6 Hz, 1H, 1'H), 5.36 (s, 2H, N3-CH_2), 4.57 (t, *J* = 6.1 Hz, 1.7 H, N^{c}-CH_2), 4.46 (t, *J* = 6.5 Hz, 0.3 H, N^{b}-CH_2), 4.36-4.43 (m, 1H, 3'H), 3.86-3.97 (m, 1H, 4'H), 3.82 (dd, *J* = 3.0 and 12.0 Hz, 1H, 5''H), 3.74 (dd, *J* = 3.5 and 12.0 Hz, 1H, 5'H), 3.51 (s, 1H, Carboranyl *H*), 2.18-2.33 (m, 2H, 2'H and 2''H), 1.96-2.15 (m, 4H, Carborane-CH_2-CH_2), 1.94 (s, 3H, 5-CH_3). ^{13}C NMR (CD_3OD, 100 MHz, δ ppm): 165.02 (=N-*C*=N-), 164.13 (C-4 C=O), 152.26 (C-2 C=O), 137.15 (C-6), 110.83 (C-5), 89.10 (C-1'), 87.37 (C-4'), 76.66 (Carboranyl *C*), 72.20 (C-3'), 62.87 (C-5'), 57.18 (Carboranyl *C*H), 53.31 (N-*C*H_2), 41.58 (C-2'), 37.36 (Carborane- *C*H_2), 34.60 (N3-*C*H_2), 30.44 (Carborane-CH_2-*C*H_2), 13.30 (5-*C*H_3); HR-MS (ESI-MS) for $C_{17}H_{33}B_{10}N_6O_5$. Calcd: 509.3516. Found: *m/z* 509.3509 $(M+H)^+$ and for $C_{17}H_{32}B_{10}N_6NaO_5$. Calcd: 531.3335. Found: *m/z* 531.3341 $(M+Na)^+$.

*4.1.11.4. 3-[1(2)-closo-1,7-Carboranylbutyltetrazol-5-ylmethyl]thymidine (**9d1/2**).* Retention time (analytical HPLC) = 17.2 min, yield = 16 mg, 62% (white wax); isomeric ratio **9d1:9d2** = 30:70. ^{1}H NMR (400 MHz, CD_3OD, δ ppm): 7.94 (s, 1H, H-6), 6.30 (t, *J* = 6.4 Hz, 1H, 1'H), 5.36 (s, 2H, N3-CH_2), 4.60 (t, *J* = 6.7 Hz, 1.5 H, N^c-CH_2), 4.51 (t, *J* = 7.1 Hz, 0.5 H, N^b-CH_2), 4.35-4.45 (m, 1H, 3'H), 3.87-3.95 (m, 1H, 4'H), 3.81 (dd, *J* = 2.9 and 12.0 Hz, 1H, 5''H), 3.73 (dd, *J* = 3.3 and 12.0 Hz, 1H, 5'H), 3.48 (s, 1H, Carboranyl *H*), 2.20-2.35 (m, 2H, 2'H and 2''H), 2.06 (t, *J* = 8.5 Hz, 0.5 H, Carborane-CH_2), 1.99 (t, *J* = 8.5 Hz, 1.5 H, Carborane-CH_2), 1.82-1.96 (m, 7H, Carborane-CH_2-CH_2, 5-CH_3), 1.83 (t, *J* = 7.4 Hz, 2H, Carborane-CH_2-CH_2). ^{13}C NMR (CD_3CN, 100 MHz, δ ppm): 163.70 (C-4 C=O), 163.58 (=N-*C*=N-), 151.62 (C-2 C=O), 136.37 and 136.12 (C-6), 110.14 (C-5), 88.26 and 88.21 (C-1'), 86.52 and 86.40 (C-4'), 77.32 (Carboranyl *C*), 71.59 and 71.49 (C-3'), 62.42 and 62.35 (C-5'), 56.73 (Carboranyl *C*H), 53.19 (N-*C*H_2), 40.70 (C-2'), 36.81 (Carborane-*C*H_2), 36.41 (N3-*C*H_2), 29.07 (N-CH_2-*C*H_2), 27.29 (Carborane-CH_2-*C*H_2), 13.21 (5-*C*H_3); HR-MS (ESI-MS) for $C_{18}H_{35}B_{10}N_6O_5$. Calcd: 523.3672. Found: *m/z* 523.3672 $(M+H)^+$ and for $C_{18}H_{34}B_{10}N_6NaO_5$. Calcd: 545.3492. Found: *m/z* 545.3411 $(M+Na)^+$.

*4.1.11.5. 3-[1(2)-Propyltetrazol-5-yl]thymidine (**11a**).* Retention time (analytical HPLC) = 19.5 min, yield = 12 mg, 71% (white amorphous solid). ^{1}H NMR (400 MHz, CD_3OD, δ ppm): 8.05 (s, 1H, H-6), 6.28 (t, *J* = 6.6 Hz, 1H, 1'H), 4.70 (t, *J* = 6.9 Hz, 2H, N^c-CH_2), 4.43 (dd, *J* = 4.7 and 8.2 Hz, 1H, 3'H), 3.90-4.00 (m, 1H, 4'H), 3.83 (dd, *J* = 3.0 and 12.1 Hz, 1H, 5''H), 3.75 (dd, *J* = 3.5 and 12.1 Hz, 1H, 5'H), 2.30 (dd, *J* = 5.2 and 6.3 Hz, 2H,

2'H and 2"H), 2.07 (hexaplet, *J* = 7.2 Hz, 2H, N-CH_2-CH_2), 1.95 (m, 3H, 5-CH_3), 0.98 (t, *J* = 7.4 Hz, 2H, propyl-CH_3). ^{13}C NMR (CD_3OD, 100 MHz, δ ppm): 164.63 (C-4 C=O), 157.85 (=N-*C*=N), 151.56 (C-2 C=O), 138.56 (C-6), 111.06 (C-5), 89.26 (C-1'), 87.50 (C-4'), 72.18 (C-3'), 62.83 (C-5'), 56.88 (N-*C*H_2), 41.45 (C-2'), 23.80 (N-CH_2-*C*H_2), 12.98 (5-*C*H_3), 11.22 (propyl-*C*H_3); HR-MS (ESI-MS) for $C_{14}H_{20}N_6NaO_5$. Calcd: 375.1393. Found: *m/z* 375.1409 $(M+Na)^+$.

*4.1.11.6. 3-[1(2)-closo-1,7-Carboranylethyltetrazol-5-yl]thymidine (**11b1/2**).* Retention time (analytical HPLC) = 16.6 min, yield = 13 mg, 56% (white wax); isomeric ratio **11b1:11b2** = 5:95. ^{1}H NMR (400 MHz, CD_3OD, δ ppm): 8.05 (s, 1H, H-6), 6.27 (t, *J* = 6.6 Hz, 1H, 1'H), 4.78 (t, *J* = 7.6 Hz, 1.9 H, N^c-CH_2), 4.55-4.65 (m, 0.1 H, N^b-CH_2), 4.43 (dd, *J* = 4.6 and 8.1 Hz, 1H, 3'H), 3.91-3.94 (m, 1H, 4'H), 3.83 (dd, *J* = 2.9 and 12.0 Hz, 1H, 5"H), 3.75 (dd, *J* = 3.5 and 12.0 Hz, 1H, 5'H), 3.59 (s, 1H, Carboranyl *H*), 2.84 (t, *J* = 7.6 Hz, 2H, Carborane-CH_2), 2.30 (t, *J* = 5.6 Hz, 2H, 2'H and 2"H), 1.96 (m, 3H, 5-CH_3). ^{13}C NMR (CD_3OD, 100 MHz, δ ppm): 164.49 (C-4 C=O), 157.87 (N-*C*=N), 151.43 (C-2 C=O), 138.53 (C-6), 110.97 (C-5), 89.19 (C-1'), 87.43 (C-4'), 73.44 (Carboranyl *C*), 72.09 (C-3'), 62.73 (C-5'), 57.59 (Carboranyl *C*H), 53.79 (N-*C*H_2), 41.36 (C-2'), 35.92 (Carborane-*C*H_2), 12.87 (5-*C*H_3); HR-MS (ESI-MS) for $C_{15}H_{28}B_{10}N_6NaO_5$. Calcd: 503.3022. Found: *m/z* 503.3067 $(M+Na)^+$.

*4.1.11.7. 3-[1(2)-closo-1,7-Carboranylpropyltetrazol-5-yl]thymidine (**11c1/2**).* Retention time (analytical HPLC) = 16.6 min, yield = 10 mg, 42% (white wax); isomeric ratio **11c1:11c2** = 5:95. ^{1}H NMR (400 MHz, CD_3OD, δ ppm): 8.05 (s, 1H, H-6), 6.28 (t, *J* =

6.6 Hz, 1H, 1'H), 4.70 (t, *J* = 6.8 Hz, 2H, N^c-CH_2), 4.35-4.48 (m, 1H, 3'H), 3.90-4.00 (m, 1H, 4'H), 3.84 (dd, *J* = 2.7 and 12.0 Hz, 1H, 5''H), 3.76 (dd, *J* = 3.3 and 12.0 Hz, 1H, 5'H), 3.51 (s, 1H, Carboranyl *H*), 2.31 (t, *J* = 6.6 Hz, 2H, 2'H and 2''H), 2.04-2.19 (m, 9H, Carborane-CH_2-CH_2), 1.96 (s, 3H, 5-CH_3). NMR (CD_3OD, 100 MHz, δ ppm): 164.63 (C-4 C=O), 157.90 (N-*C*=N), 151.47 (C-2 C=O), 138.52 (C-6), 110.99 (C-5), 89.19 (C-1'), 87.43 (C-4'), 76.55 (Carboranyl *C*), 72.10 (C-3'), 62.74 (C-5'), 57.08 (Carboranyl *C*H), 54.18 (N-*C*H_2), 41.35 (C-2'), 34.44 (N-CH_2-*C*H_2), 30.34 (Carborane-*C*H_2), 12.88 (5-*C*H_3); HR-MS (ESI-MS) for $C_{16}H_{30}B_{10}N_6NaO_5$. Calcd: 517.3179. Found: *m/z* 517.3128 $(M+Na)^+$.

*4.1.11.8. 3-[1(2)-closo-1,7-Carboranylbutyltetrazol-5-yl]thymidine (**11d1/2**).* Retention time (analytical HPLC) = 17.8 min, yield = 11 mg, 45% (white wax); isomeric ratio **11d1:11d2** = 10:90. ^{1}H NMR (400 MHz, CD_3OD, δ ppm): 8.05 (s, 1H, H-6), 6.28 (t, *J* = 6.6 Hz, 1H, 1'H), 4.73 (t, *J* = 6.8 Hz, 1.6 H, N^c-CH_2), 4.56 (t, *J* = 6.9 Hz, 0.4 H, N^b-CH_2), 4.43 (dd, *J* = 4.6 and 8.2 Hz, 1H, 3'H), 3.94 (dd, *J* = 3.0 and 6.2 Hz, 1H, 4'H), 3.84 (dd, *J* = 2.9 and 12.0 Hz, 1H, 5''H), 3.76 (dd, *J* = 3.5 and 12.0 Hz, 1H, 5'H), 3.47 (s, 1H, Carboranyl *H*), 2.30 (t, *J* = 5.7 Hz, 2H, 2'H and 2''H), 1.84-2.10 (m, 9 H, Carborane-CH_2-CH_2-CH_2, 5-CH_3). ^{13}C NMR (CD_3OD, 100 MHz, δ ppm): 164.59 (C-4 C=O), 157.93 (N-*C*=N), 151.53 (C-2 C=O), 138.57 (C-6), 111.05 (C-5), 89.28 (C-1'), 87.52 (C-4'), 77.58 (Carboranyl *C*), 72.20 (C-3'), 62.84 (C-5'), 57.01 (Carboranyl *C*H), 54.67 (N-*C*H_2), 41.46 (C-2'), 37.23 (N-CH_2-*C*H_2), 29.66 (Carborane-*C*H_2), 27.89 (Carborane-CH_2-*C*H_2), 12.99 (5-*C*H_3); HR-MS (ESI-MS) for $C_{17}H_{33}B_{10}N_6O_5$. Calcd: 509.3516. Found: *m/z* 509.3457

$(M+H)^+$, for $C_{17}H_{32}B_{10}N_6NaO_5$. Calcd: 531.3335. Found: *m/z* 531.3365 $(M+Na)^+$ and for $C_{17}H_{32}B_{10}N_6KO_5$. Calcd: 547.3074. Found: *m/z* 547.3081 $(M+K)^+$.

4.2. Enzymatic studies

4.2.1. Phosphorylation Transfer Assays (PTAs). Recombinant hTK1 was expressed and purified using the bacterial expression system [BL21(DE3) pLysS transformed with the vector hTK1-pET-14b], as described previously [29,30]. The activity of the enzyme with 100 µM dThd was determined to be 441 nmol dThd monophosphate formed per minute and per mg of hTK1 protein.

For PTA studies, dThd and the N3-substituted dThd derivatives were dissolved in DMSO (100 mM concentration) and further diluted with water to produce stock solutions of concentrations (0.4 mM). The phosphorylation transfer assays were carried out as described previously with minor modifications [29-31]. The reaction mixtures contained 100 µM nucleoside and 100 µM ATP (with a small fraction of 0.13 µM [γ-^{32}P]ATP (10 µCi/µL), 25 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 a total volume of 20 µL. In all reactions, the final concentration of DMSO was set to < 1%. The reaction mixtures were incubated at 37 °C for 20 min in the presence of 135 ng of enzyme. Following the incubation period, the enzyme was inactivated by heating for 5 min at 99 °C. The reaction mixtures were centrifuged and 2 µL sample portions were spotted on PEI–cellulose TLC plates (EMD Chemicals Inc.). The TLC plates were developed in a solvent system containing isobutyric acid:ammonium hydroxide:water (66:1:33) over a period of 6 h. The radiolabeled spots were visualized by placing the TLC plates on a BioMax XAR film

(Kodak) overnight and developing the films using a X-ray film developer (Tiba M6B, Series VI B Rapid processor; Commonwealth X-Ray Inc.). The spot intensities of the phosphorylated compounds were calculated with Adobe Photoshop (Adobe Systems Incorporated, USA).

4.2.2. Enzyme Kinetic Studies (K_m and k_{cat} values). The enzyme kinetics studies were carried out using the protocol described above for PTAs with varying substrate concentrations. The reaction mixtures contained 0.1-300 µM nucleoside and 100 µM ATP (with a small fraction of 0.325 µM [γ-^{32}P]ATP (Perkin Elmer), 25 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 125mM KCl, 10 mM DTT, and 0.5 mg/mL bovine serum albumin (BSA). The kinetic parameters were calculated with the enzyme kinetic module of SigmaPlot 12 (Systat Software Inc, USA) using the Michaelis-Menton equation.

4.2.3. Phosphorylation Transfer Assays for in vitro Metabolite Determination. Compound **7f** was dissolved in DMSO (10 mM concentration) and further diluted with water to a concentration of 1 mM. The phosphorylation transfer assay was carried out as described in section 4.2.1 with minor modifications. The reaction mixture contained 250 µM **7f** and 1 mM ^{31}P-ATP, 25 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 a total volume of 2 mL. The reaction mixture was incubated at 37 °C for 24 h in the presence of 1.5 µg of enzyme. Following the incubation period, the enzyme was inactivated by heating for 5 min at 99 °C. The reaction mixture was centrifuged and the supernatant were analyzed by LR-ESI-MS to detect the presence of dTMP and **7f**-monophosphate.

4.3. Solubility Studies.

The protocol for the solubility studies was generously provided by Dr. Hardeep Singh Saluja (College of Pharmacy, The Southwestern Oklahoma State University). Calibration curves for known compound concentrations were generated using area under the curves (AUCs) from analytical HPLC data. These calibration curves were used to determine the unknown quantities of compounds dissolved in PBS buffer (pH 7.4). The quantity of ~ 5 mg of **N5-2OH**, **3e, 3f, 9b1/2-9d1/2**, or **11b1/2-11d1/2** were added to micro centrifuge tubes containing PBS (0.2-1 mL, pH 7.4). The micro centrifuge tubes were placed in a shaker overnight and centrifuged at 3300 rpm for 5 min. The solutions were passed through 0.2 µm filters and analyzed by HPLC to obtain the AUCs corresponding to amount of the drug soluble in PBS buffer.

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some of the phosphoryl transfer assays. The authors also thank Dr. Hardeep Singh Saluja from College of Pharmacy, The Southwestern Oklahoma State University, for providing the protocol for the solubility studies and Dr. Robert W. Curley, Jr., for his help with the ^{1}H-^{1}H NOESY NMR studies.

Appendix A. Supplementary material

Supplementary data related to this article can be found online, at …….. Data include original MS-, ^{1}H-NMR-, and ^{13}C-NMR spectra, HPLC chromatograms, ^{1}H-^{1}H NOESY NMR data, HPLC methods, as well as methods for hTK1 purification and the determination of hTK1 concentrations.

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Figure and Scheme Legends

Figures

Figure 1: Structures of N5 and N5-2OH.

Figure 2: Phosphorylation rates of dThd, N5-2OH and N3-substituted dThd analogues with hTK1. Assay products were separated by PEI–cellulose TLC and quantified by *β*-radiography. (**A**). Compounds **3e**, **3f** and **7e-7g**; (a) dThd monophosphate (dTMP), (b) compound-monophosphates (**N5-2OH**, **3e**, **3f** and **7e-7g**) and (c) monophosphorylated degradation products, presumably dTMP. (**B**). Compounds **8**, **9a, 9b1/2-9d1/2**, (a) dTMP and (b) compound-monophosphates (**8**, **9a, 9b1/2-9d1/2**). (**C**). Compounds **10**, **11a, 11b1/2-11d1/2**, (a) dTMP and (b) compound-monophosphates (**11a, 11b1/2-11d1/2**)

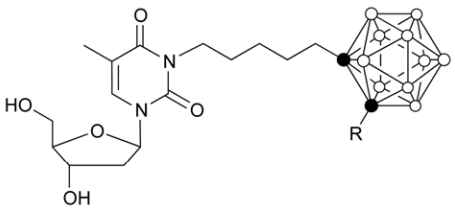
Schemes

Scheme 1: Synthesis of N3-amidine-type dThd derivatives (**3a-3f**).

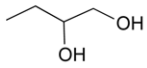
Scheme 2: Synthesis of N3-guanidine-type dThd derivatives (**7a-7g**).

Scheme 3: Synthesis of N3-tetrazolylmethyl-type dThd derivatives (**9a, 9b1/2-9d1/2**).

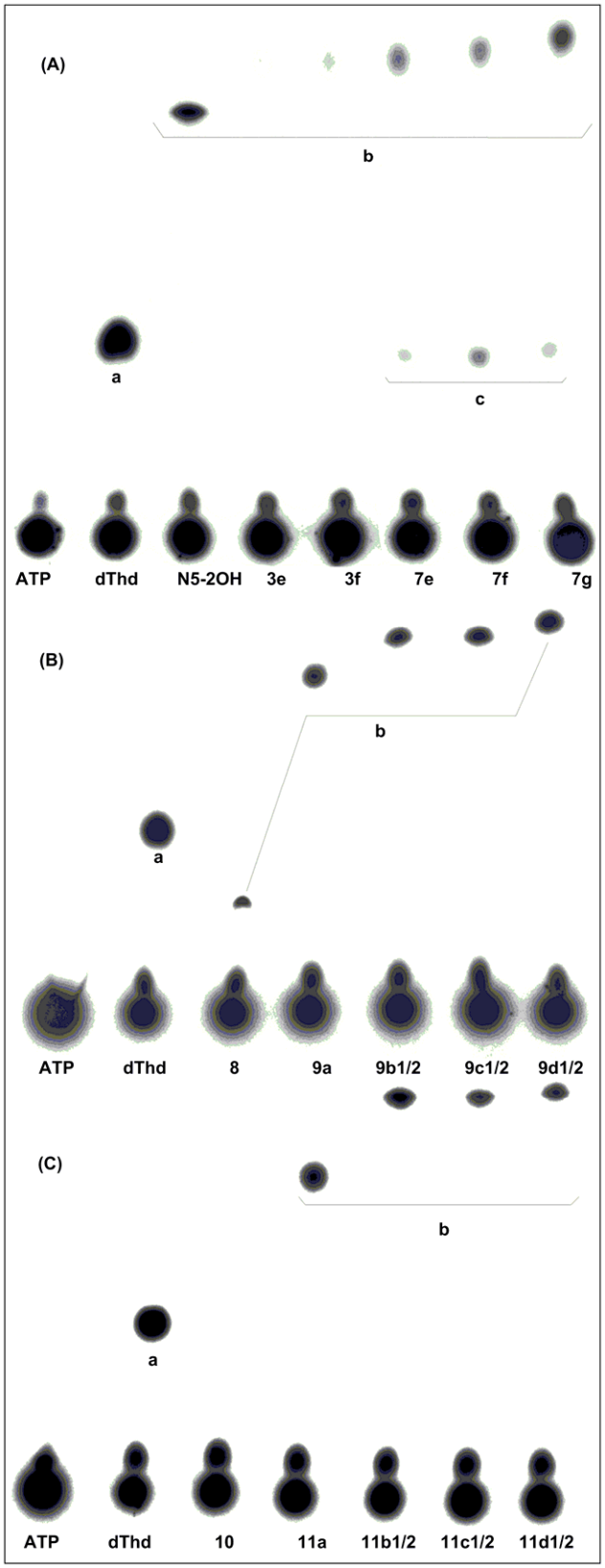
Scheme 4: Synthesis of N3-tetrazolyl-type dThd derivatives **(11a, 11b1/2-11d1/2**).



N5: **R = H**

N5-2OH: R = 

○ = BH
● = C


(A)
b
a
c
ATP
dThd
N5-2OH
3e
3f
7e
7f
7g
(B)
b
a
ATP
dThd
8
9a
9b1/2
9c1/2
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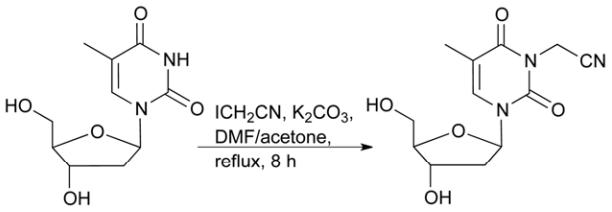
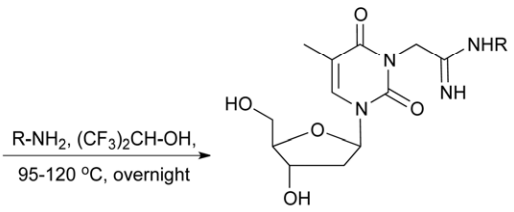
10
11a
11b1/2
11c1/2
11d1/2



1 **2** (78%)



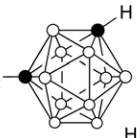
3a (24%): R = -$(CH_2)_2CH_3$

3b (45%): R = -$(CH_2)_3CH_3$

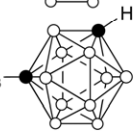
3c (79%): R = -C_6H_5

3d (81%): R = -$CH_2C_6H_5$

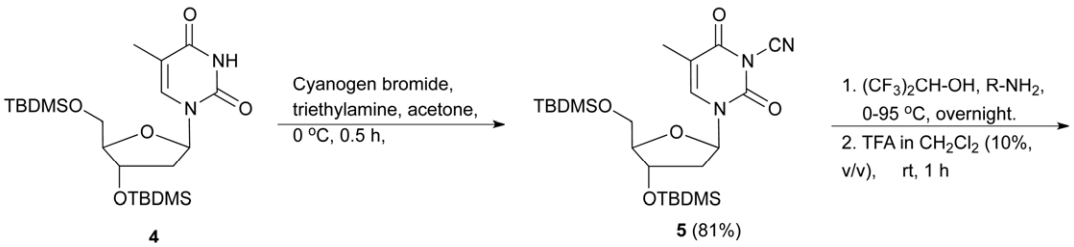
3e (7%): R = —$(CH_2)_2$

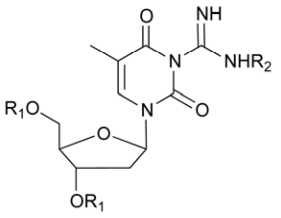


3f (10%): R = —$(CH_2)_3$



○ = BH
● = C





6a (95%): R_1 = TBDMS
7a (40%): R_1 = H
R_2 = -$(CH_2)_2CH_3$

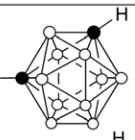
6b (95%): R_1 = TBDMS
7b (46%): R_1 = H
R_2 = -$(CH_2)_3CH_3$

6c (93%): R_1 = TBDMS
7c (70%): R_1 = H
R_2 = -C_6H_5

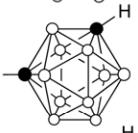
6d (99%): R_1 = TBDMS
7d (80%): R_1 = H
R_2 = -$CH_2C_6H_5$

○ = BH
● = C

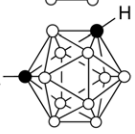
6e (32%): R_1 = TBDMS
7e (69%): R_1 = H
R_2 = $-(CH_2)_2$

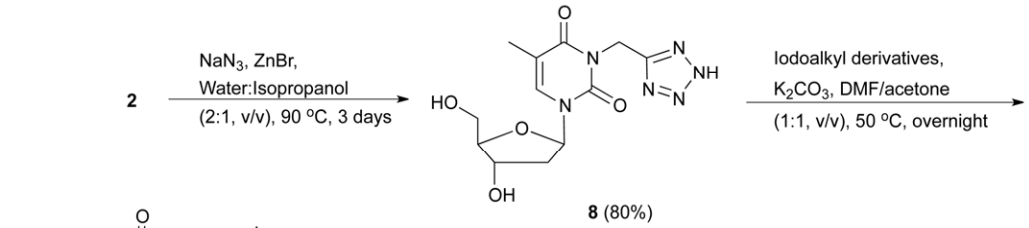


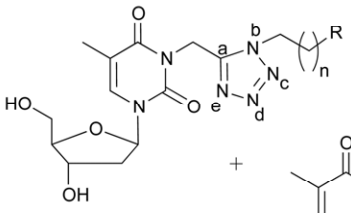
6f: R_1 = TBDMS
7f (29%): R_1 = H
R_2 = $-(CH_2)_3$

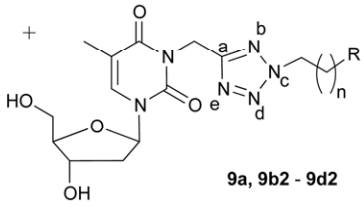


6g (38%): R_1 = TBDMS
7g (80%): R_1 = H
R_2 = $-(CH_2)_4$

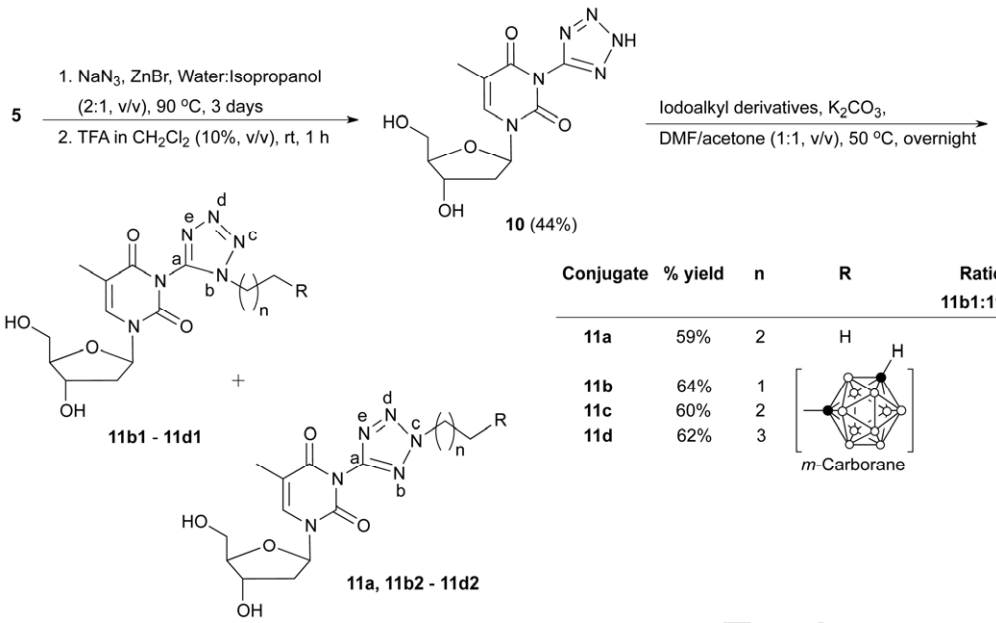








Conjugate	% Yield	n	R	Ratio of isomers 9b1:9b2 - 9d1:9d2
9a	59%	2	H	-
9b	64%	1	*m*-Carborane	20:80
9c	60%	2	*m*-Carborane	15:85
9d	62%	3	*m*-Carborane	30:70



Conjugate	% yield	n	R	Ratio of isomers 11b1:11b2 - 11d1:11d2
11a	59%	2	H	-
11b	64%	1	*m*-Carborane	5:95
11c	60%	2	*m*-Carborane	5:95
11d	62%	3	*m*-Carborane	10:90

Table 1. Relative hTK1 phosphorylation rates (rPRs) of N3-substituted dThd analogues.

Compounds	rPR (%) ± SD[1]	Compounds	rPR (%) ± SD[1]
dThd	100	**7f**	15.5 ± 1.9
N5-2OH	46.3 ± 14.0	**7g**	27.3 ± 3.0
3a	1.9 ± 0.5	**8**	39.5 ± 3.6
3b	2.3 ± 0.4	**9a**	55.7 ± 5.2
3c	11.3 ± 0.9	**9b1/2**	52.4 ± 5.8
3d	16.8 ± 0.8	**9c1/2**	58.2 ± 1.2
3e	3.0 ± 0.2	**9d1/2**	63.7 ± 3.4
3f	5.6 ± 0.5	**10**	0.0 ± 0.0
7a	0.0 ± 0.0	**11a**	57.6 ± 8.5
7b	1.2 ± 0.9	**11b1/2**	55.6 ± 3.2
7c	1.9 ± 1.3	**11c1/2**	42.9 ± 5.1
7d	14.9 ± 2.1	**11d1/2**	42.5 ± 5.8
7e	16.3 ± 1.0		

[1]Data represent the means of three replicates ± SD.

Table 2. Enzymatic properties of selected N3-substituted dThd analogues.

Compounds	K_m (μM) ± SD[1]	k_{cat} (s^{-1}) ± SD[1]	rk_{cat}/K_m (%)
dThd	4.9 ± 1.2	0.46 ± 0.03	100.0
N5-2OH	41.5 ± 10.0	0.42 ± 0.03	10.8
9a	37.3 ± 7.0	0.45 ± 0.03	12.9
9b1/2	31.8 ± 8.4	0.44 ± 0.03	14.9
9c1/2	55.2 ± 15.8	0.39 ± 0.04	7.6
9d1/2	56.3 ± 13.2	0.47 ± 0.04	8.9
11a	14.0 ± 2.4	0.34 ± 0.02	26.0
11b1/2	35.6 ± 10.8	0.35 ± 0.03	10.7
11c1/2	79.9 ± 36.4	0.37 ± 0.06	5.0
11d1/2	17.4 ± 5.4	0.32 ± 0.02	19.7

[1]Data represent the means of three replicates ± SD

Table 3. Solubilities of the **N5-2OH**, **3e**, **3f**, **9b1/2-9d1/2** and **11b1/2-11d1/2** in PBS at pH 7.4 at 25 °C.

Compounds	**PBS pH 7.4**[1]	**Compounds**	**PBS pH 7.4**[1]
N5-2OH	7-10 µg/mL	**9d1/2**	33.9 ± 1.4 µg/mL
3e	>19.5 mg/mL	**11b1/2**	42.6 ± 1.0 µg/mL
3f	>15 mg/mL	**11c1/2**	25.4 - 26.3 µg/mL[2]
9b1/2	133.1 ± 5.2 µg/mL	**11d1/2**	13.3 – 13.8 µg/mL*
9c1/2	84.2 ± 4.1 µg/mL		

[1]Standard deviations are based on three experiment, *Data represent duplicate measurements