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Design, Synthesis, and Antiviral Evaluation of Chimeric Inhibitors of HIV Reverse Transcriptase

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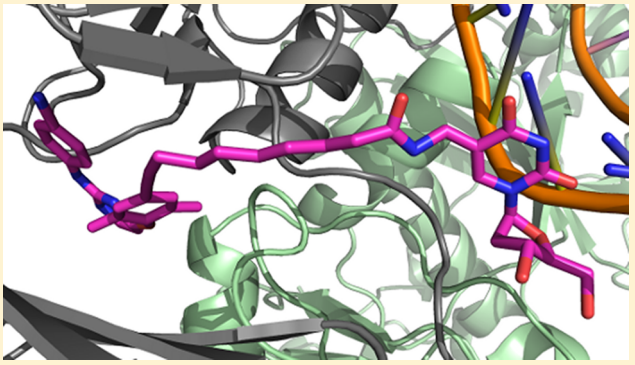
Supporting Information

ABSTRACT: In a continuing study of potent bifunctional anti-HIV agents, we rationally designed a novel chimeric inhibitor utilizing thymidine (THY) and a TMC derivative (a diarylpyrimidine NNRTI) linked via a polymethylene linker (ALK). The nucleoside, 5′-hydrogen-phosphonate (H-phosphonate), and 5′-triphosphate forms of this chimeric inhibitor (THY-ALK-TMC) were synthesized and the antiviral activity profiles were evaluated at the enzyme and cellular level. The nucleoside triphosphate (**11**) and the H-phosphonate (**10**) derivatives inhibited RT polymerization with an IC_{50} value of 6.0 and 4.3 nM, respectively. Additionally, chimeric nucleoside (**9**) and H-phosphonate (**10**) derivatives reduced HIV replication in a cell-based assay with low nanomolar antiviral potencies.



KEYWORDS: *HIV, reverse transcriptase, nucleoside H-phosphonate, NNRTI, synthesis*

The concept of linking two parent molecules that yield one high affinity chimeric compound was introduced by Jencks et al.[1] and successfully implemented to discover chimeric ligands exhibiting low nanomolar binding affinities for various proteins.[2] The topic of connecting different HIV inhibitors targeting two distinct sites has grown in interest over the last 20 years and has recently been reviewed.[3] The design of chimeric inhibitors containing both nucleoside and non-nucleoside moieties was first suggested by Nanni et al. based on the observation of the close proximity (10−15 Å) of the respective nucleoside reverse transcriptase inhibitor (NRTI) and non-nucleoside reverse transcriptase inhibitor (NNRTI) binding sites in HIV-1 reverse transcriptase (RT).[4] Further rationale includes mechanistic studies establishing that the two sites could be simultaneously occupied[5,6] and studies on the mechanism of synergistic inhibition of RT by NRTIs and NNRTIs.[7−9] Bifunctional inhibitors targeting HIV-1 RT involve two distinct classes: Those that join the two drugs via a cleavable linker, in which both drugs could be released into the cytoplasm once the bifunctional compound enters the cell,[10−15] and those that utilize noncleavable linker to combine NRTIs and NNRTIs with the expectation of synergistic inhibitory effects.[16−19]

Our group published several designs and syntheses of bifunctional NRTI-linker-NNRTI compounds from the latter class of noncleavable linker compounds including 2′,3′-dideoxy-2′,3′-didehydrothymidine (d4T) as the NRTI, a poly(ethylene glycol) (PEG) linker, and different NNRTI moieties such as phenethylthiazolyl (PETT) derivative HI-236[20] and tetrahydroimidazobenzodiazepinone (TIBO).[21] Most recently, we have reported a bifunctional inhibitor (d4T-4PEG-TMC) design with low nanomolar activity against RT.[22] This inhibitor includes d4T and a PEG linker as the earlier inhibitors, while a diarylpyrimidine (DAPY) derivative, referred as TMC-derivative in Figure 1, was especially chosen as the NNRTI portion due to its flexibility and high antiviral potency against wild-type (WT) and drug-resistant HIV strains.

The TMC-derivative that we used in that bifunctional design resembles the FDA-approved TMC125 (Figure 1) without the bromine and amino substituents on the pyrimidine ring for ease of chemical synthesis. Since NRTIs are nucleoside analogue prodrugs that require intracellular phosphorylation to their respective pharmacologically active triphosphate forms by the host cellular kinases, we synthesized the 5′-triphosphate form of the bifunctional nucleoside analogue (d4TTP-4PEG-TMC, **12**, Figure 2) and evaluated its inhibitory activity using biochemical experiments.[22] Indeed, **12** exhibited a low nanomolar anti-HIV activity in RT polymerization inhibition assays that was more potent than both individual parent drugs and thus indicating synergistic binding as supported by the molecular modeling studies. On the basis of this earlier study, the purpose of the current work was to further exploit new linker and nucleoside components in the design (i.e., hydrophobic alkyl linker). This information would aid in an understanding of pharmacophore space and development of structure−activity relationships (SAR) by targeting both the RT polymerization active site

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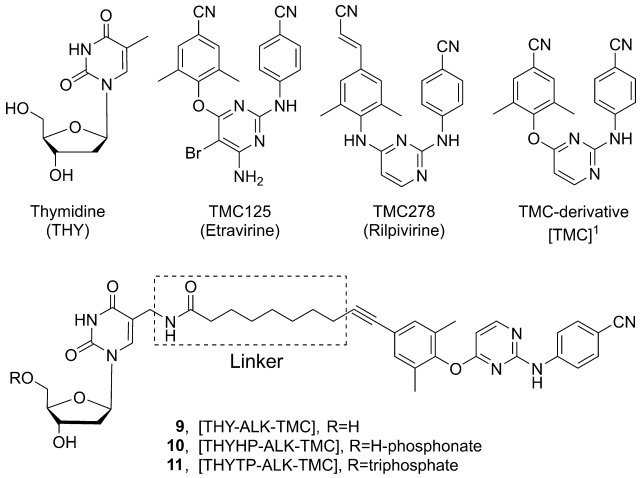

Figure 1. Structures of thymidine, various TMC derivatives, and [THY-ALK-TMC] derivatives.[1] For simplicity, we refer to [TMC-derivative] as TMC in text.

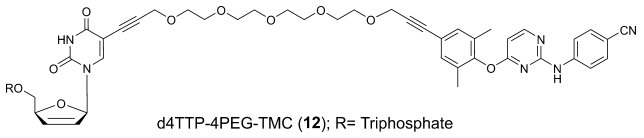

Figure 2. Structure of d4TTP-4PEG-TMC (**12**) bifunctional inhibitor.

and the NNRTI-binding pocket (NNBP) simultaneously. Guided by our previous findings,[22] the same unsubstituted TMC-derivative was chosen for targeting the NNBP due to its conformational flexibility and high antiviral potency. We decided to select thymidine (THY) for the nucleotide binding site of HIV-1 RT with the aim of simplifying the chemical synthesis. Additionally, as reported by others, the C5-substituted 2′-deoxyribonucleoside analogues could still serve as substrates for human deoxyribonucleoside kinases (dNKs).[23] Human dNKs are responsible for the monophosphorylation of various antiviral nucleoside analogues.[24,25] This initial step of monophosphorylation is considered to be the rate limiting step compared to the subsequent phosphorylation steps catalyzed by deoxynucleoside mono- and diphosphate kinases to afford the active triphosphate metabolites responsible for antiviral activity via inhibition of HIV-1 RT and termination of DNA chain growth.[26]

Once the two end fragments have been selected that target their respective binding sites on the RT complex, the linker length, chemical entity, and the attachment points are determined through computational molecular modeling along with the accessible information from various X-ray crystal structures. We designed a polymethylene linker (ALK) that would attach and span the hydrophobic tunnel distance between the two fragments and avoid steric clash with the protein. A representative model of the ternary complex of THYHP-ALK-TMC (**10**), RT, and primer-template was generated using the X-ray structures of RT/TMC278[27] and RT/TTP/primer-template[28] (Figure 3A). Additionally, the linker includes amide functionality for chemical feasibility with an attachment to the nucleobase from the C5 position due to its anticipated low interference with base pairing. In light of the fact that the former bifunctional prototype exhibited excellent potency against HIV-1 RT, we retained the para position of wing I of TMC scaffold for the linker attachment,[22]

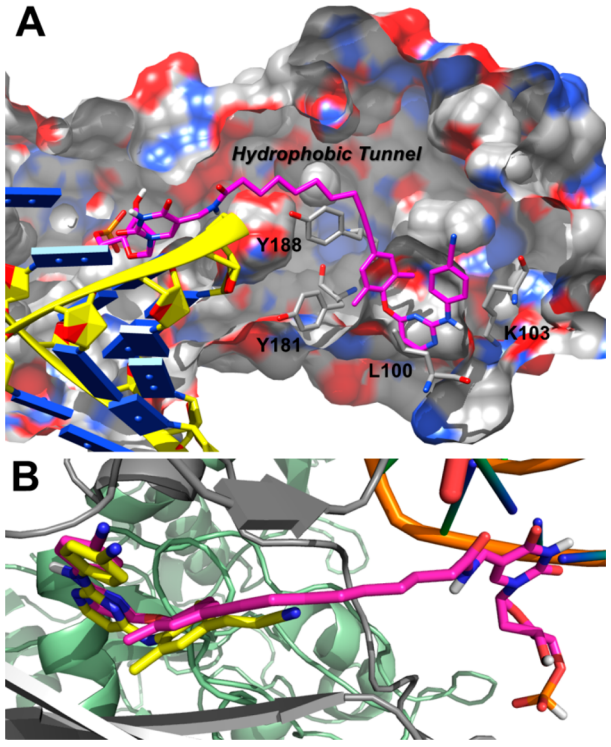

Figure 3. Molecular modeling predicts binding of THYHP-ALK-TMC (**10**). (A) The polymethylene linker is predicted to extend from the NNRTI binding pocket toward the active site through a hydrophobic tunnel, similar to the tunnel identified in the TMC278 bound structure (pdb ID: 2ZD1).[27] It would allow proper base-pairing of the nucleoside portion with the templating A. (B) Overlay of TMC278[27] (yellow) and our modeled H-phosphonate derivative **10** (magenta) bound structure showing a similar placement of the wing I of the NNRTI toward the hydrophobic tunnel.

where the linker protrudes into a hydrophobic tunnel in the NNBP that opens to the active site for THY binding (Figure 3B). Therefore, the synthetic targets of the present study were the chimeric THY-ALK-TMC (**9**, Figure 1) nucleoside derivative with the hydrophobic chemical linkage and the corresponding triphosphate THYTP-ALK-TMC (**11**, Figure 1). These two target compounds could be evaluated for inhibitory activity against RT at the biochemical level and anti-HIV activity at the cellular level. Several previous studies designing bifunctional inhibitors have only evaluated the nucleoside version of the compound in cellular assays.[18,29] However, since the mode of action requires phosphorylation of the nucleoside fragment for achieving desired efficacy in cellular assays, inhibitors that are impaired in their cellular activation would not demonstrate efficacy even though the phosphorylated form if produced might be able to bind and inhibit RT. For this reason, in an effort to develop SAR for these bifunctional inhibitors, we focused our studies on first synthesizing the metabolically active 5′-triphosphate form of THY-ALK-TMC (THYTP-ALK-TMC, **11**, Figure 1) and evaluating its antiviral activity via in vitro incorporation assays catalyzed by HIV-1 RT. Those bifunctional inhibitors exhibiting potency could then be evaluated as the nucleoside version in HIV cell culture.

The nucleoside part of the desired molecule (**4**) is synthesized first starting from commercially available thymidine **1**, which was protected to **2** and brominated at the 5-methyl position followed by conversion to 5-azidomethyl derivative using sodium azide (NaN_3) to afford **3**. The hydrogenation of

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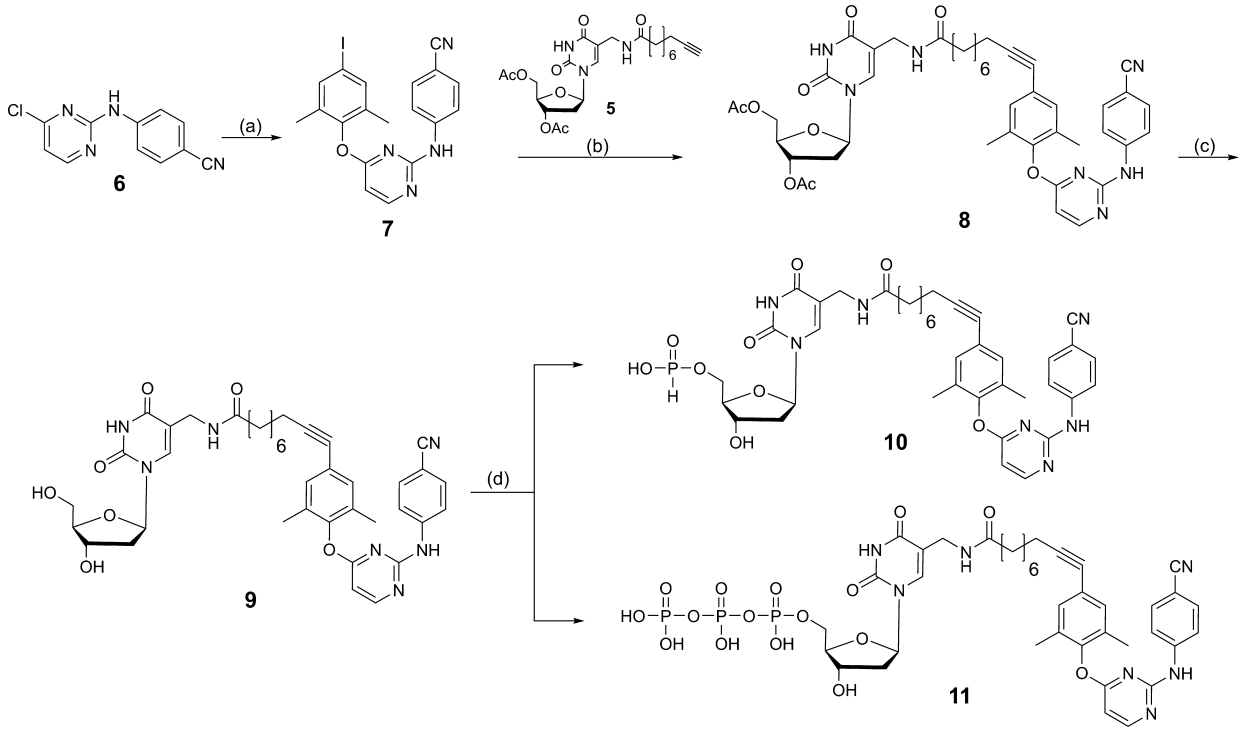
Scheme 1. Synthesis of 5′-*H*-Phosphonate and 5′-Triphosphate of THY-ALK-TMC Chimeric Derivatives[a]



[a]Reagents and conditions: (a) 4-iodo-2,6-dimethylphenol, Cs_2CO_3, DMF, Δ, 85%. (b) Compound 5, $Pd(PPh_3)_4$ (10 mol %), CuI (50 mol %), THF, DMF, 95%. (c) MeOH, K_2CO_3, water, 66%. (d) (i) 2-Chloro-1,3,2-benzodioxaphosphorin-4-one, pyridine, DMF, 0 °C to rt; (ii) bis(tributylammonium) pyrophosphate; (iii) I_2; (iv) 1 M Et_3NH^+ HCO_2^-, 35%.

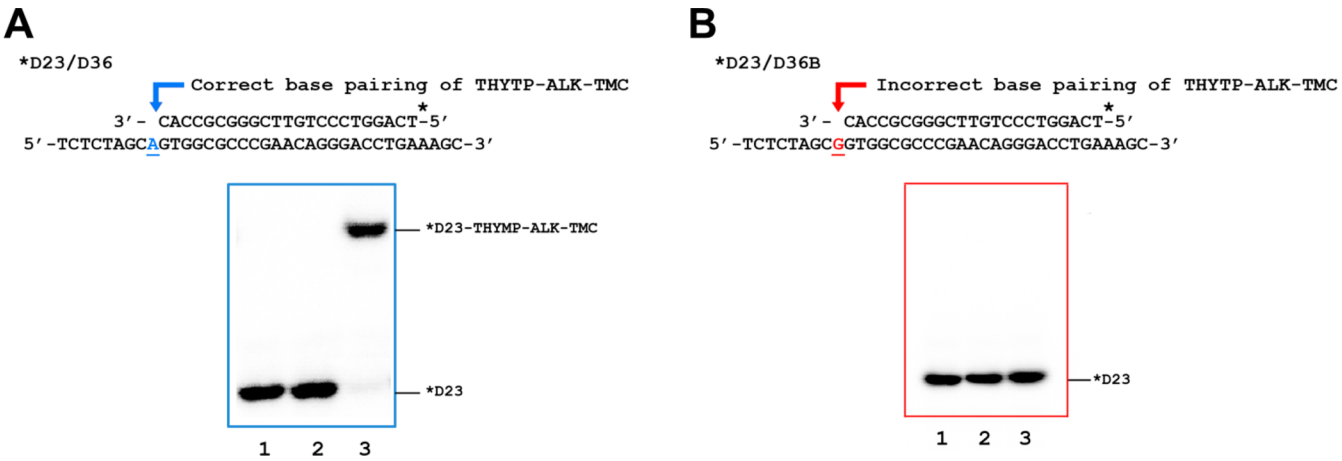


Figure 4. Incorporation and potent HIV-1 RT inhibition of THYTP-ALK-TMC (11) is base specific. (A) The sequence of D23/D36 P/T for correct base pairing used in biochemical assays. THYTP-ALK-TMC serves as a substrate for HIV-1 RT catalyzed polymerization when using a primer for TTP incorporation. Lane 1 represents the 0 time point, and lane 2 serves as a negative control lacking Mg^{2+}. Lane 3 represents the incorporated chimeric-TP (indicated as *D23-THYTP-ALK-TMC) product band by HIV-1 RT. (B) The sequence of D23/D36B P/T for incorrect base pairing used in biochemical assays. THYTP-ALK-TMC does not serve as a substrate for HIV-1 RT when using a primer for dCTP incorporation. Lane 1 represents the 0 time point, and lane 2 serves as a negative control lacking Mg^{2+}. Lane 3 shows the lack of an incorporated chimeric-TP product band.

the azide group using palladium on charcoal led to the corresponding primary amine derivative **4**. Subsequently, 9-decynoic acid was chosen to build the appropriate length for the linker and connected to the 5-aminomethyl group on the pyrimidine ring of **4** to afford compound **5** in around 85% isolated yield (Supporting Information Scheme S1). The NNRTI moiety was then synthesized starting from the known pyrimidine derivative **6**,[30] which underwent nucleophilic substitution in high yield to **7** with 4-iodo-2,6-dimethylphenol[31] by use of cesium carbonate as base (Scheme 1). Subsequent Sonogashira coupling with compound **5** afforded the desired coupled product **8** in high overall yields. Finally, the deacetylation of **8** using methanol and potassium carbonate gave the chimeric nucleoside inhibitor (THY-ALK-TMC, **9**) in good yield. The purified nucleoside **9** was converted to its triphosphate derivative (THYTP-ALK-TMC, **11**) via the procedure of Ludwig and Eckstein.[32] Interestingly, besides the expected product **11**, the 5′-H-phosphonate derivative

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(THYHP-ALK-TMC, **10**) was also formed as a byproduct in the reaction due to the presence of a trace amount of water reacting with 2-chloro-1,3,2-benzodioxaphosphorin-4-one as reported earlier.[33]

To investigate the incorporation of THYTP-ALK-TMC (**11**) by HIV-1 RT, single-turnover incorporation experiments were conducted using two sets of primer/template (P/T) sequences. We first utilized D23/D36mer P/T that would provide a correct base pairing for **11** as it serves as a THY analogue. Indeed, a fully elongated primer including the incorporated chimeric monophosphate, indicated as D23-THYMP-ALK-TMC, was observed with a significantly higher migration distance than a natural single deoxynucleotide incorporation band size in Figure 4A. A similar type of gel migration analysis was also performed using D23, D23-THYMP-ALK-TMC, and D24 (TTP incorporated product) primers, and the assay illustrated a major migration distance difference between the natural nucleotide incorporated primer (D24) and the chimeric nucleotide incorporated primer as shown in Figure S1, Supporting Information. Additionally, we explored the effect of base pairing on the incorporation of chimeric inhibitor **11** via utilizing the other D23/D36Bmer P/T, which allows a dC base pairing opposite to a template dGMP. Notably, we observed an absence of elongated primer band formation as shown in Figure 4B. Magnesium chloride lacking negative control experiments (lanes 2) were also performed and absence of any elongated product formation illustrates the essential requirement for the presence of divalent metal ions for catalysis. These observations unambiguously indicate that THYTP-ALK-TMC **11** is incorporated in a correct base-pairing manner, and the significance of Mg^{2+} ion for catalysis. Most importantly, the altered mobility of the D23-THYMP-ALK-TMC band on the gel is a strong evidence for the chimeric nucleotide interacting with both sites of HIV-1 RT and serving as a substrate for nucleotide incorporation. We also used the same experiment for THYHP-ALK-TMC (**10**) and no product formation was observed as expected supporting the above notion (Data not shown).

We next turned our attention to determine the antiviral potency of compounds **10** and **11** via a steady-state competition assay comprising inhibition of TTP incorporation opposite template A into a radiolabeled D23/D36mer P/T (Figure 5). The IC_{50} values for in vitro inhibition of RT were determined, and the results are summarized in Table 1. The metabolically active THYTP-ALK-TMC **11** and the 5′-H-phosphonate derivative **10** exhibited an IC_{50} value of 6.0 and 4.3 nM, respectively. These potency values were 2-fold and 3-fold more enhanced than the parent TMC-derivative (IC_{50} = 13 nM). One possible reason for the observed increase in antiviral potency for the chimeric compounds relative to the TMC-derivative could be a result of lowering the free energy of binding via linking the two compounds.[1] Moreover, the linked fragments could form additional hydrogen bonds, hydrophobic, and van der Waals interactions with the protein that could improve the binding affinity of these compounds. These results are in agreement with previous observations of reactions run under the same conditions containing the bifunctional inhibitor **12**.[22] Expanding our SAR studies by adopting an aliphatic linker retains low nanomolar inhibitory activity and 2000- to 3000-fold improvement in potency relative to an NRTI like d4T (IC_{50} = 13 000 nM; data is adapted from ref 22).

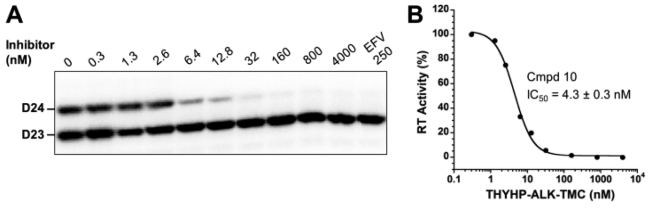

Figure 5. Inhibitory effects of THYHP-ALK-TMC (**10**) on TTP incorporation assay. (A) A representative gel showing a reduction in product formation by the increasing amounts of inhibitor concentration as indicated. Efavirenz (EFV) was used as a positive control at 250 nM concentration. (B) Percent RT activity loss was plotted against various concentrations of **10** to generate a half maximal inhibitory concentration (IC_{50}) of 4.3 ± 0.3 nM.

Table 1. HIV-1 RT Inhibitory Activity of Chimeric Derivatives 10 and 11

compd	IC_{50} (nM)[a]
TMC derivative[b]	13 ± 3
THYHP-ALK-TMC (**10**)	4.3 ± 0.3
THYTP-ALK-TMC (**11**)	6 ± 1

[a]Data derived from three independent experiments. IC_{50} is the concentration of compound that inhibits HIV-1 RT activity by 50%. [b]Data was adapted from ref 22.

After demonstrating significantly high antiviral potencies at the biochemical level, the next step was the evaluation of the nucleoside version of the chimeric inhibitor **9** in a cell culture assay. The purified THY-ALK-TMC (**9**), d4T, and ddC as positive controls for the NRTI portion in addition to TMC-derivative and TMC120 as the NNRTI moiety controls were evaluated for inhibition of HIV-1 (IIIB) replication in MT-2 cell culture using an MTT assay according to a previously published protocol.[34] The results are shown in Supporting Information Table S1. The chimeric nucleoside **9** exhibited a 30 times higher inhibitory activity than d4T (EC_{50} = 3.6 μM), and a similar CC_{50} value of 2.2 μM compared to ddC (CC_{50} = 4 μM) and TMC120 (CC_{50} = 1.9 μM). We also tested THYHP-ALK-TMC (**10**) in the cell culture assay, even though the 5′-H-phosphonate group comprises a single negative charge that could hinder the cell membrane penetration through passive diffusion.[35] Additionally, the specific organic anion transporters, which may play a critical role in the uptake of negatively charged drugs,[36] could facilitate the transport of compound **10** into the cell. Indeed, the 5′-H-phosphonate derivative **10** exhibited a moderate 2-fold increase in EC_{50} value and yet 3-fold less cytotoxicity than the nucleoside derivative **9.** The 2-fold reduction in activity for compound **10** was expected considering the aspects of cell permeability mentioned above. Therefore, the observed results suggest that compound **10** gets transported into the cell and illustrates its activity albeit in less quantity.

Moreover, it must be noted here that 5′-H-phosphonate derivative of an NRTI, 5′-*H*-phosphonate-3′-azido-3′-deoxythymidine (AZT H-phosphonate, Nikavir), has been approved by the Russian Ministry of Health for the prevention and treatment of HIV-infected patients as a consequence of lower toxicity profile and a longer half-life than AZT while inducing much slower selection of drug-resistant viruses.[37] A recent study suggests that although AZT H-phosphonate is structurally similar to AZT-monophosphate (AZTMP), it might be more resistant to extra and/or intracellular dephosphorylation and could penetrate through cellular membranes due to its less

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polar nature by possessing only a single negative charge unlike a monophosphate group with two negative charges.[38] Overall, the chimeric inhibitors **9** and **10** have exhibited potencies 16- to 30-fold higher than d4T with a selectivity index of 18 and 27, respectively. Consequently, our findings suggest that these compounds present a hybrid cellular inhibition profile that is in line with the current FDA approved NRTIs and NNRTIs. Taken into consideration all previously reported data, our chimeric inhibitors especially the H-phosphonate derivative **10** support the notion of unique, valuable drug candidates for potential use in HIV treatment.

In conclusion, we have utilized molecular modeling to design novel chimeric HIV-1 RT inhibitors, which are as potent as our previously designed bifunctional inhibitor.[22] This study expands our SAR investigations on the bifunctional designs that target two distinct sites on RT and suggests that THYHP-ALK-TMC (**10**) derivative could be a valuable candidate for prodrug design to enhance its antiviral potency while reducing the cellular toxicity all at once. There are several options that we can explore for the pronucleotide approach such as generating phosphoroamidate[39] or alkyl and aryl phosphoester[40] derivatives of the 5′-H-phosphonate. Lastly, potential therapeutic drugs based on the chimeric inhibitor designs are specific to HIV-1 RT and would presumably not interfere with any human polymerases such as human mitochondrial DNA polymerase γ and thus would result in fewer side effects relative to NRTIs. We are also currently exploring targeted drug delivery through nanogel formulations carrying the triphosphate forms of these chimeric inhibitors.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic procedures, spectral data, cell culture and in vitro assay conditions, and modeling procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

HIV, human immunodeficiency virus; WT RT, wild-type reverse transcriptase; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, nonnucleoside reverse transcriptase inhibitor; dNTP, deoxynucleoside triphosphate

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