4-Oxo-4,7-dihydrothieno[2,3-*b*]pyridines as Non-Nucleoside Inhibitors of Human Cytomegalovirus and Related Herpesvirus Polymerases

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A novel series of 4-oxo-4,7-dihydrothieno[2,3-*b*]pyridine-5-carboxamides have been identified as potential antivirals against human herpesvirus infections resulting from human cytomegalovirus (HCMV), herpes simplex virus type 1 (HSV-1), and varicella-zoster virus (VZV). Compounds **10c** and **14** demonstrated broad-spectrum inhibition of the herpesvirus polymerases HCMV, HSV-1, and VZV. High specificity for the viral polymerases was observed compared to human α polymerase. The antiviral activity of **10c** and **14**, as determined by plaque reduction assay, was comparable or superior to that of existing antiherpes drugs, ganciclovir (for HCMV) and acyclovir (for HSV-1 and VZV). Drug resistance to compound **14** correlated to point mutations in conserved domain III of the herpesvirus DNA polymerase, but these mutations do not confer resistance to existing nucleoside therapy. In addition, compound **14** maintained potent antiviral activity against acyclovir-resistant HSV-1 strains. Substitution to the pyridone nitrogen (N7) was found to be critical for enhanced in vitro antiviral activity.

Introduction

Diseases resulting from infections by viruses of the Herpesvirus family continue to play a significant role in patient morbidity and diminished individual quality of life. Especially troublesome among viruses of the family is human cytomegalovirus (HCMV). Although generally benign in the immunocompetent host, HCMV infection is associated with clinical symptoms such as pneumonia, retinitis, and graft rejection in the immunocompromised, as well as congenital birth defects in neonates.[1] The prevalence of other herpesviruses such as herpes simplex virus (HSV) types 1 and 2 and varicella-zoster virus (VZV) is widespread among the human population. Infection by HSV is responsible for labial and genital herpes, while VZV on primary infection causes chicken pox.[2] Reactivation of latent VZV infection is associated with herpes zoster (shingles), a highly debilitating illness characterized by persistent neuropathic pain.[3] Existing therapies for the management of herpesvirus infections have been dominated by nucleoside analogues such as acyclovir, ganciclovir, and famciclovir. The nucleoside-based antivirals require phosphorylation by a viral specific thymidine kinase and subsequently cellular enzymes to afford an active drug form, the nucleoside triphosphate. The resulting triphosphate acts as a substrate for the viral DNA polymerase, competing with the binding of natural 2′-deoxynucleoside triphosphate, and leads to partial or complete chain termination after incorporation.[4] As a consequence, these therapies have a limited spectrum of utility among viruses of the family because both enzymes must recognize the drug as a substrate. The requirement for two viral-specific enzymes in the pathway for inhibition by the nucleoside analogues has also led to increased risk of resistance especially in the growing immunocompromised population.[5] Many existing therapies are also hampered by significant drug-associated toxicities that limit their duration of use. As a consequence, there is an increasing realization of the need for less toxic, orally bioavailable, broad-spectrum agents to address herpesvirus infections in the growing immunocompromised population, particularly in AIDS patients and organ transplant recipients. The identification of a broad-spectrum, non-nucleoside antiviral that directly inhibits the viral DNA polymerase therefore would offer clear advantages toward existing therapies.

The herpesvirus DNA polymerase represents a compelling molecular target to identify broad-spectrum agents because of the high degree of homology among the family.[6] Seven regions (I−VII) have been identified as being highly conserved by most members of the family. Through the characterization of HSV polymerase mutants it has been suggested that regions II and III play a critical role in substrate recognition.[7] Indeed, point mutations within these regions have been associated with resistance to established therapies such as acyclovir and ganciclovir.[5,6,8]

Recently, we have reported a novel series of 4-oxo-1,4-dihydroquinoline (DHQ) carboxamides, represented by PNU-181128, PNU-181465, and PNU-183792, that demonstrated broad-spectrum inhibition of HCMV, HSV-1, and VZV polymerases (Figure 1).[9] These non-nucleoside agents were found to be mixed competitive inhibitors of nucleoside binding, however, not cross-resistant to ganciclovir-resistant HCMV or acyclovir-

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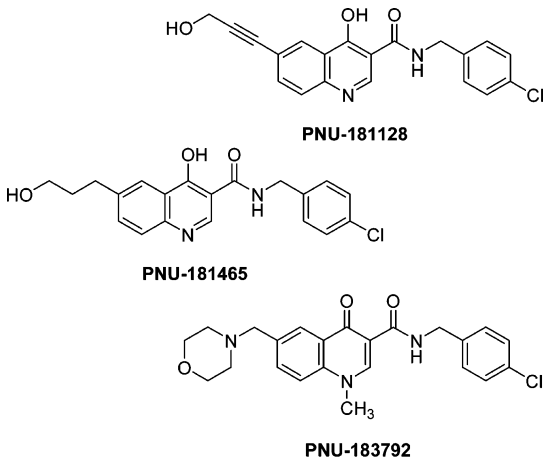


Figure 1. Lead 4-oxo-1,4-dihydroquinoline carboxamides.

resistant HSV-1 mutants. Structure−activity relationship studies around these lead structures as well as related naphthalene carboxamides suggested that the ortho orientation of the carboxamide and the hydroxy (or oxo) group was critical for activity and that the C6 substituent played a significant role toward enhanced potency.[9,10]

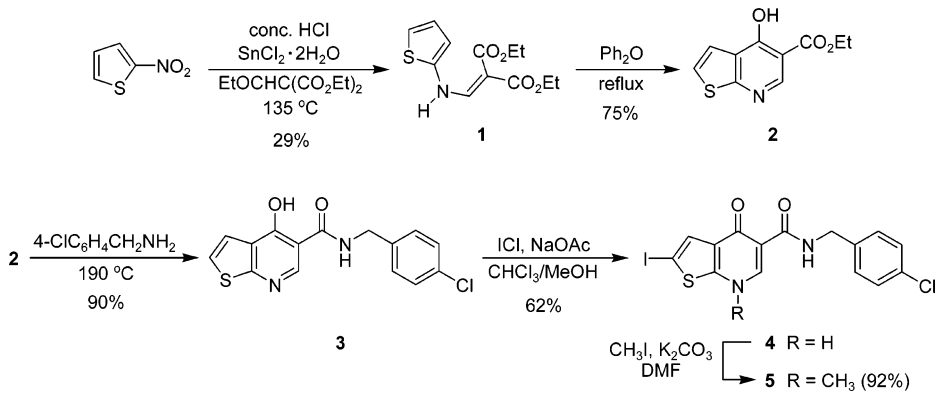
We were intrigued by the possibility that the central heterocycle may serve an organizational role for these three pharmacophoric elements and that other isosteric structures might play a similar role. Noteworthy among possible isosteres to the quinoline ring is the thieno[2,3-*b*]pyridine heterocycle (Scheme 1, **2**) in which a thiophene now replaces the fused benzene ring. Although the ring size has been contracted, the increased atomic radius of sulfur versus carbon still allows for spatial superpositioning between the C6 position of the quinoline ring and the C2 position of the thieno[2,3-*b*]-pyridine ring. This report examines the viability of such an isosteric replacement toward maintained herpes DNA polymerase inhibition. Initial structure−activity relationships have been characterized for the C2 substituent (hydroxyalkyl, hydroxyalkynyl, or morpholin-4-ylmethyl) as well as the significance of pyridone nitrogen substitution.

Chemistry

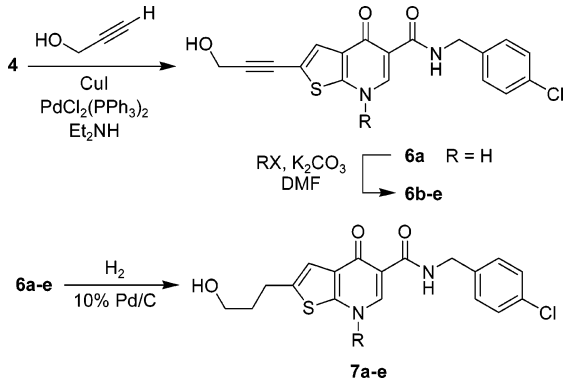
The synthesis of the thieno[2,3-*b*]pyridine nucleus was accomplished by following an adaptation of existing Gould-Jacob cyclization methodology employing enamine **1**, Scheme 1.[11] Reduction of 2-nitrothiophene with tin(II) chloride[12] afforded the unstable amine that was immediately condensed with diethyl ethoxymethylenemalonate to provide enamine **1**. Subsequent thermal cyclization in degassed diphenyl ether afforded thieno[2,3-*b*]pyridine carboxylate ester **2**. Conversion of the ester to the desired 4-chlorobenzyl amide **3** was accomplished by simply heating a mixture of **2** and the amine at 190 °C with removal of ethanol. To elaborate the C2 position of the thienopyridine ring, amide **3** was regioselectively iodinated at this position with iodine monochloride to provide **4**.

Iodide **4** served as the common intermediate for the synthesis of analogues with diversity at both the C2 and the N7 positions. In the first series of compounds, the 3-hydroxypropyl and 3-hydroxy-1-propynyl substituents were maintained at the C2 position while the N7 substituent was surveyed. Sonogashira coupling between iodide **4** and propargyl alcohol provided alkyne **6a** (Scheme 2). The pyridone nitrogen readily underwent regioselective alkylation with a variety of electrophiles (iodoethane, **b**; 2-bromopropane, **c**; 2-bromoethanol, **d**; 2-bromo-*N*,*N*-diethylethylamine, **e**) to afford alkylated products **6b**−**e**. Subsequent hydrogenation of **6a**−**e** afforded the corresponding 3-hydroxypropyl analogues **7a**−**e**. In the converse approach, the N7 substituent was maintained as methyl, and the tether length to the hydroxy moiety at the C2 position was surveyed in the alkyl and alkynyl series. The shortest homologue, hydroxymethyl, was prepared from **4** by palladium-catalyzed carbonylation in the presence of methanol to afford methyl carboxylate ester **8** (Scheme 3). Subsequent reduction of the ester with lithium aluminum hydride followed by alkylation of the pyridone nitrogen with iodomethane provided hydroxymethyl compound **10a**. Preparation of the two-carbon homologue, 2-hydroxyethyl, required initial alkylation of the pyridone nitrogen; therefore, iodide **4** was converted to compound **5** with iodomethane (Scheme 1). Subsequent coupling of **5** with tributylvinylstannane under Stille conditions yielded vinyl derivative **11** (Scheme 4). The sequence of hydroboration−oxidation employing **11** and 9-borabicyclo-[3.3.1]nonane afforded hydroxyethyl compound **10b**. The remaining homologues at the C2 position were prepared by Sonogashira coupling between iodide **5** and a corresponding hydroxyalkyl acetylene (propargyl alcohol, 3-butyn-1-ol, and 4-pentyn-1-ol) to afford alkynes **12a**−**c** (Scheme 5). Hydrogenation of the resulting alkynes provided hydroxyalkyl derivatives **10c**−**e**.

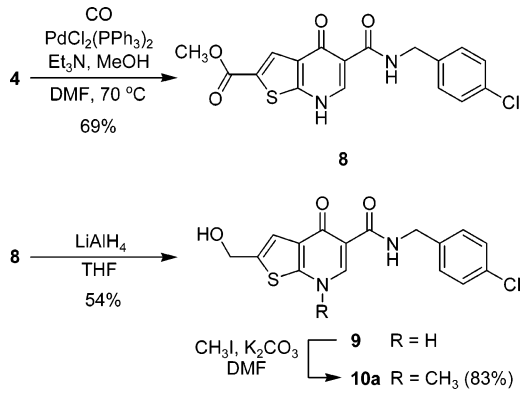
Scheme 1



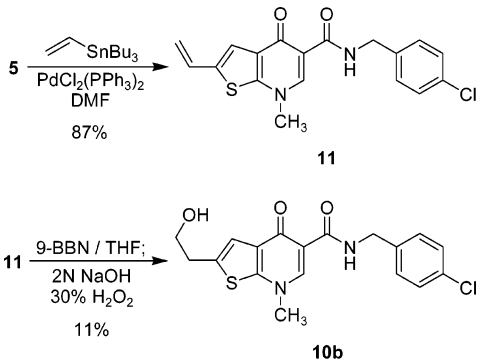
Scheme 2



Scheme 3



Scheme 4



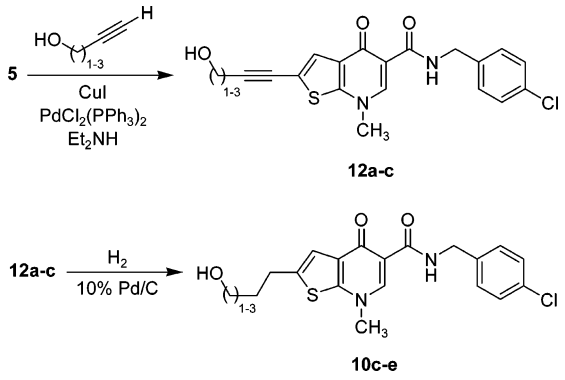
Synthesis of the corresponding 2-(morpholin-4-ylmethyl) analogue in the thieno[2,3-*b*]pyridine series proved to be straightforward utilizing Mannich chemistry. Carboxamide **3** was reacted with morpholine in the presence of acetic acid to provide **13** (Scheme 6). Subsequent alkylation of the pyridone nitrogen with iodomethane provided the targeted analogue **14**.

Results and Discussion

For primary screening, the ability of compounds to inhibit HCMV polymerase activity was assessed by determining the reduction in ^{3}H-labeled nucleotide (dTTP) incorporation into a primer template (biotinylated oligo(dT)16-poly(dA)) through a scintillation proximity assay. The antiviral activity of select compounds was then evaluated in cell culture plaque reduction assays employing HCMV (Davis strain).

The first milestone to be realized was whether the quinoline ring system found in previous lead compounds could be replaced by the thieno[2,3-*b*]pyridine nucleus and still maintain inhibitory activity against a viral polymerase. To answer this question, the direct structural analogues to PNU-181128 and PNU-181465 were prepared and tested, **6a** and **7a**, respectively (Table 1). As anticipated, both compounds demonstrated inhibition of HCMV polymerase; however, antiviral activity in cell culture was poor (IC_{50} > 20 μM) compared to quinoline derivatives PNU-181128 (IC_{50} = 1.1 μM) and PNU-181465 (IC_{50} = 0.4 μM). Nonetheless, these results suggested that the thieno[2,3-*b*]pyridine series warranted further examination. Further structural modi-

Scheme 5



Scheme 6

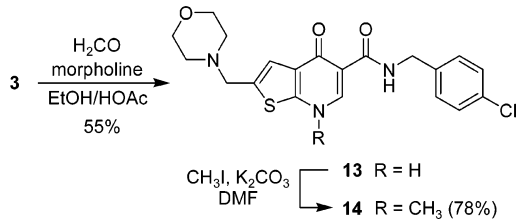
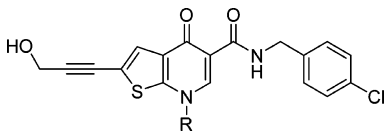
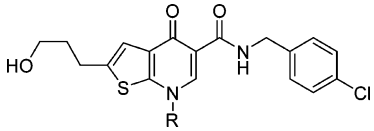
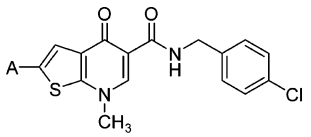


Table 1. Influence of N7 Substituent on HCMV Polymerase Inhibition and HCMV Antiviral Activity

R	compd	HCMV polymerase IC_{50} (μM)[a]	HCMV antiviral IC_{50} (μM)[a,b]
			
H	**6a**	2.7 ± 0.2	>20.0
CH_3	**12a**	1.0	1.4 ± 0.8
CH_2CH_3	**6b**	1.8	1.7 ± 0.4
$CH(CH_3)_2$	**6c**	4.2	2.7 ± 0.2
CH_2CH_2OH	**6d**	1.1 ± 0.2	2.3
$CH_2CH_2NEt_2$	**6e**	1.8 ± 0.2	3.8 ± 0.3
			
H	**7a**	2.2 ± 0.1	>20.0
CH_3	**10c**	0.40	0.6 ± 0.0
CH_2CH_3	**7b**	0.67	2.3 ± 0.1
$CH(CH_3)_2$	**7c**	2.4	1.3 ± 0.0
CH_2CH_2OH	**7d**	0.75	1.8
$CH_2CH_2NEt_2$	**7e**	0.81	0.9 ± 0.2

[a] Value represents the mean IC_{50} ± SEM derived from two determinations (single determination where SEM is absent). [b] Determined by plaque reduction assay (Davis strain).

Table 2. HCMV Polymerase Inhibition for C2-Hydroxyalkyl and Hydroxyalkynyl Homologues



10a-e A = $(CH_2)_nOH$
12a-c A = C≡C-$(CH_2)_{n-2}OH$

n	compd	IC_{50} (μM)[a]	compd	IC_{50} (μM)[a]
1	**10a**	18.5		
2	**10b**	4.0		
3	**10c**	0.40	**12a**	1.0
4	**10d**	0.43	**12b**	9.5
5	**10e**	9.7	**12c**	22.0

[a] IC_{50} value derived from a single determination. 15−25% historical average SEM for controls.

fication focused on the N7 (pyridone nitrogen) substituent while maintaining the 3-hydroxypropyl and 3-hydroxy-1-propynyl substituents at C2. As shown in Table 1, substitution was well tolerated at N7 in both series with respect to HCMV polymerase inhibition. Simple alkyl as well as functionalized alkyl (hydroxyethyl and *N*,*N*-diethylaminoethyl) substitution demonstrated good inhibition with only alkyl branching adjacent to the ring (isopropyl, **6c** and **7c**) appearing to be detrimental. In contrast to **6a** and **7a**, HCMV antiviral activity for the substituted series correlated well with HCMV polymerase inhibition showing a greater than 10-fold improvement in antiviral activity over the unsubstituted analogues. The stark difference between the cellular antiviral activity of **7a** and the corresponding alkylated derivatives such as **10c** may be associated with the intrinsic Lewis acidity of the 4-hydroxythieno[2,3-*b*]-pyridine (**3**, pK_a = 6.8), which may impede cellular transport. Alkylation at nitrogen therefore eliminates the acidic proton and affords a neutral molecule.

The observation that the inhibitory activity of the alkylated derivatives against HCMV polymerase was comparable or superior to that of **6a** or **7a** also sheds light on the physiologically significant tautomeric form of the molecules at the binding site and a refinement of the role the C4 oxygen substituent plays as a pharmacophoric element. Although the relative position of the keto/enol tautomer population for **6a** and **7a** under physiological or assay conditions is unknown,[13] the potency of the alkylated series, where the enol tautomer population has been excluded, suggests that the keto tautomer is the dominant species binding to the polymerase in the cases of **6a** and **7a**. In addition, it suggests that the oxygen substituent at the 4-position of the ring serves a common pharmacophoric role as either an inter- or intramolecular hydrogen bond acceptor in both series.

A more noticeable structural impact on the HCMV polymerase inhibition was realized as the length and degrees of freedom to the tether between the thienopyridine C2 position and the terminal hydroxyl group was surveyed (Table 2). For this series of analogues, the 7-methyl group was maintained for consistency. A parabolic relationship was observed in the hydroxyalkyl series (**10a**−**e**) as the distance was increased. Optimal positioning of the hydroxyl group was in the region of three (**10c**, 3-hydroxypropyl) to four (**10d**, 4-hydroxybutyl) methylenes. Both compounds **10c** and **10d** exhibited similar potency in whole cells against HCMV (IC_{50} = 0.6 and 0.8 μM, respectively). In the alkynyl series (**12a**−**c**), the shortest homologue examined (**12a**, 3-hydroxy-1-propynyl) proved to be the most potent against HCMV polymerase. Mirroring the behavior observed in the hydroxyalkyl series, activity diminished as the tether length was increased. Consistent with the previous experience in the DHQ carboxamide series, morpholin-4-ylmethyl derivative **14** also demonstrated inhibition of HCMV polymerase (IC_{50} = 1.1 μM).

Compounds **10c** and **14** were subsequently evaluated against an expanded set of herpesvirus polymerases (HSV-1 and VZV) and human DNA polymerases to assess the activity spectrum and specificity (Table 3). Compound **10c** demonstrated broad-spectrum inhibition of the herpesvirus polymerases with activity comparable to that observed for the 4-hydroxyquinoline PNU-181465. High specificity for the viral polymerases was observed compared to human α polymerase. The morpholin-4-ylmethyl derivative **14** demonstrated a similar profile of viral DNA polymerase specificity (human α, γ, and δ evaluated). The broad-spectrum profile of these compounds against the polymerases was similarly observed when **10c** and **14** were examined for antiviral effects against HCMV, HSV-1, and VZV, employing plaque reduction assays. The activity of **10c** and **14** was comparable to that of ganciclovir against HCMV and acyclovir against HSV-1, while it was significantly more potent against VZV compared to acyclovir. A high therapeutic index was also observed with respect to cytotoxicity in both human foreskin fibroblast (HFF) and Vero cells (CC_{50} > 100 μM).

To more fully characterize the mechanism of antiviral activity derived from the 4-oxo-4,7-dihydrothieno[2,3-*b*]pyridine series, enzyme kinetics and the consequence of polymerase mutation were assessed for the representative compound **14**. Enzyme kinetics were examined with respect to inhibition of dTTP incorporation into the primer template with HCMV polymerase. In contrast to previously examined 4-hydroxyquinoline antivirals that demonstrated mixed competitive behavior,[9a] **14** was found to be a competitive inhibitor of substrate binding to the polymerase (K_i = 1.0 μM) (Figure 2). Previously, we have reported the identification of HSV-1 and HSV-2 resistant mutants to the 4-oxo-1,4-dihydroquinoline antiviral class.[14] The resulting mutants carried a single point mutation to the viral DNA polymerase characterized by a V823A (HSV-1) or V826A (HSV-2) amino acid change. These amino acid residues correspond to conserved region III of the herpes DNA polymerase wherein valine is conserved in six (HSV-1, HSV-2, HCMV, VZV, Epstein−Barr virus, and HHV-8 (human herpes virus 8)) of the eight human herpes viruses. In the case of HCMV, serial passage experiments were unsuccessful in isolating a resistant strain. A mutant resistant to members of the 4-oxo-1,4-dihydroquinoline antiviral class was identified after transfecting cells with HCMV polymerase carrying two point mutations (V823A/V824L) corresponding to the valine residues in the region analogous to that of HSV-1. The three resulting mutant strains were subsequently used to further verify the mode of antiviral activity exhibited by compound **14**. Indeed, plaque reduction assays conducted with

Table 3. Broad-Spectrum Activity of **10c** and **14** Compared to 4-Oxo-1,4-dihydroquinolines and Established Therapies

	polymerase IC_{50} (μM)[a]						antiviral IC_{50} (μM)[e]			CC_{50} (μM)[g]	
compd	HCMV	HSV-1	VZV	α	γ	δ	HCMV	HSV-1	VZV	HFF	Vero
10c	0.4	0.5	0.46	>40.0	nd[h]	nd[h]	0.6	3.0	0.09	>100	>100
14	1.1[b]	1.2	0.77	>40.0	>40.0	>40.0	0.8[f]	3.7	0.77	>100	>100
PNU-181465[c]	0.4	0.3	0.07	>40.0	>40.0	>40.0	1.0	7.4	0.17	>100	>100
PNU-183792[c]	0.7	0.6	0.37	>40.0	>40.0	>40.0	0.9	3.5	0.34	>100	>100
ganciclovir							1.3	nd[h]	nd[h]	>100	>100
acyclovir							>20.0	2.1	8.1	>100	>100
foscarnet	2.5	nd[h]	nd[h]	>20.0	< 0.28	>20.0					
aphidicolin	0.4	0.5	0.60	2.6	2.6	>40.0					
AZT-TP[d]	22.1	3.3	5.8	13.4	12.0	2.3					

[a] IC_{50} value derived from a single determination unless otherwise noted. 15−25% historical average SEM for controls. [b] $n = 5$, SEM ± 0.4 μM. [c] Reference 9a. [d] AZT-TP = zidovudine triphosphate. [e] Determined by plaque reduction assay (HCMV, Davis strain; HSV-1, KOS strain; VZV, Webster strain). Value represents the mean IC_{50} value derived from two determinations unless otherwise noted. Average SEM ± 25%. [f] $n = 6$, SEM ± 0.3 μM. [g] CC_{50}, 50% cellular cytotoxicity, derived from single determination. [h] nd: not determined.

Table 4. Conferred Resistance to **14** after Induced Mutation to Conserved Region III of Herpes Polymerases and Activity against Drug-Resistant HSV-1 Isolates

	antiviral plaque reduction IC_{50} (μM)[c]										
	HCMV Ad169		HSV-1 KOS					HSV-1 Patton		HSV-2	
compd	WT[a]	V823A[b]	WT	V823A	AraA^{r}13	PAA^{r}5	PFA^{r}2	WT	BW^r	WT	V826A
14	1.5	6.2	5.6	> 50.0	2.1	0.8	2.5	5.4	2.5	0.8	> 50.0
acyclovir			3.5	4.4	> 10.0	> 10.0	> 10.0	5.4	> 10.0	2.4	2.8
ganciclovir	0.8	0.8									

[a] WT: wild-type parental virus. [b] Strain contains two residue mutations (V823A/V824L). [c] Value represents the mean IC_{50} value derived from two determinations, average SEM ± 25%.

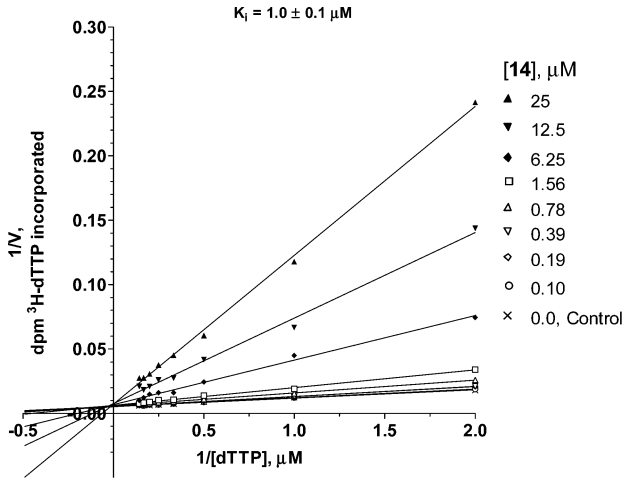


Figure 2. Enzyme kinetics analysis for compound **14**.

these mutant strains resulted in a 4- to 10-fold increase in IC_{50} for compound **14** with respect to wild type (Table 4). The resulting mutant strains, however, did not demonstrate a reduced sensitivity to the nucleoside analogues ganciclovir (HCMV) and acyclovir (HSV-1 and HSV-2). The results further support that compound **14** elicits an antiviral response through binding to and subsequent inhibition of the viral DNA polymerase. It also suggests that **14** makes critical contacts within conserved domain III of the polymerase (implicated in nucleotide binding); however, the binding mode is distinct from that of nucleoside analogues such as ganciclovir and acyclovir. The marked difference in antiviral profile from the nucleoside analogues is also evident when **14** was evaluated against acyclovir-resistant HSV-1 KOS strains (AraA^{r}13, PAA^{r}5, and PFA^{r}2) and an HSV-1 Patton strain (BW^r).[15] The acyclovir-resistant strains were found to be equally sensitive (or in some cases hypersensitive) to compound **14** compared to the corresponding wild-type strains.

Conclusion

A novel series of 4-oxo-4,7-dihydrothieno[2,3-*b*]pyridine-5-carboxamides have been identified as non-nucleoside inhibitors of herpesvirus DNA polymerases including HCMV, HSV-1, and VZV. Alkylation of the pyridone nitrogen (N7) affords compounds that demonstrate in vitro antiviral activity against these three viruses. These results indicate that indeed the thieno-[2,3-*b*]pyridine ring system is a competent isosteric replacement for the quinoline ring found in previously reported 4-oxo-1,4-dihydroquinoline antivirals such as PNU-183792. Compounds **10c** and **14** exhibited broad-spectrum antiviral activity against HCMV, HSV-1, and VZV with activity comparable to that of ganciclovir against HCMV and that of acyclovir against HSV-1. Both compounds were found to be significantly more potent against VZV when compared to acyclovir. In addition, compound **14** was shown not to be cross-resistant with existing nucleoside analogues. As a class, 4-oxo-4,7-dihydrothieno[2,3-*b*]pyridine-5-carboxamides offer an exciting platform to identify antivirals that may address the unmet medical needs still present resulting from herpesvirus infection.

Experimental Section

All reagents were obtained from commercial suppliers and used without further purification. *N*,*N*-Dimethylformamide (DMF) was of anhydrous grade. Column chromatography was performed using either 40S or 40M Biotage KP-Sil silica prepacked cartridges. Solvents for extraction and chromatography were of HPLC grade. Brine refers to a saturated aqueous solution of sodium chloride. Chemical shifts for ^{1}H NMR and ^{13}C NMR are given in ppm (δ); multiplicities are indicated by s (singlet), d (doublet), t (triplet), q (quartet), qt (quintet), m (multiplet), or bs (broadened singlet). Data for mass spectra analysis are reported in the form *m*/*z* (intensity relative to base = 100). Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected.

Diethyl 2-((2-Thienylamino)methylene)malonate (1). 2-Nitrothiophene (85%, 100 g, 0.77 mol) was suspended in

concentrated hydrochloric acid (550 mL) in a 2 L, three-necked, round-bottom flask, and the mixture was heated to 40−45 °C with vigorous stirring. Stannous chloride dihydrate (350 g, 1.55 mol, 2.0 equiv) was added portionwise to the mixture, maintaining the reaction temperature between 45−50 °C by immersion in an ice bath. After the addition was complete, the cooling bath was removed, and the mixture was allowed to cool to 30−35 °C over 1 h. The mixture was further cooled to below 5 °C, and the precipitate was filtered and washed with concentrated hydrochloric acid (20 mL). The brown solid was dried in a stream of air for 20 min, washed with diethyl ether (50 mL), and dried for an additional 30 min. The above procedures were repeated, and the two lots were combined to afford 169.6 g (0.52 mol) of the hexachlorostannate salt as a brown solid. The resulting salt was dissolved in water (3 L) and filtered through a Celite plug. In 500 mL portions, the solution was neutralized with ammonia hydroxide (50 mL) followed by 2 N sodium hydroxide (50 mL) and was extracted with CH_2Cl_2 (3 × 100 mL). The combined organic layers were immediately mixed with diethyl ethoxymethylenemalonate (106 mL, 0.52 mol) and dried (Na_2SO_4). The solution was filtered and concentrated in vacuo (40 °C). The crude product was purified by column chromatography (heptane/2-propanol; 30/1, 20/1, 10/1) and mixed fractions were further purified (heptane/2-propanol; 50/1, 30/1). The product-containing fractions were concentrated and the enamine was crystallized from hexane/ethyl acetate (300 mL, 9/1) to afford 72.2 g as an off-white solid. Mixed fractions from the chromatography containing diethyl ethoxymethylenemalonate were concentrated and crystallized from hexane/ethyl acetate (100 mL, 9/1) to afford 21.2 g as a yellow solid. The filtrate from these crystallizations was concentrated and purified by column chromatography employing the same conditions as above. The product-containing fractions were concentrated and crystallized from hexane/ethyl acetate (100 mL, 9/1) to afford an additional 11.0 g as a white solid. The above procedure afforded a combined yield of 104.4 g (29%). Mp 39−40 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.90 (br, 1 H), 8.06 (s, 1 H), 7.15 (dd, J = 5.4, 1.5 Hz, 1 H), 6.97 (dd, J = 3.7, 1.5 Hz, 1 H), 6.91 (dd, J = 5.3, 3.7 Hz, 1 H), 4.15 (br, 4 H), 1.24 (t, J = 7.1 Hz, 6 H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 166.7, 164.7, 152.6, 144.2, 126.5, 118.7, 114.9, 93.4, 59.6, 59.5, 14.2. Anal. ($C_{12}H_{15}NO_4S$) C, H, N, S.

Ethyl 4-Hydroxythieno[2,3-*b*]pyridine-5-carboxylate (2). Enamine **1** (25.0 g, 92.8 mmol) and diphenyl ether (250 mL) were combined and degassed (freeze−pump−thaw). The reaction mixture was heated to reflux for 10 min. The mixture was allowed to cool to room temperature and was then purified by column chromatography (CH_2Cl_2; CH_2Cl_2/methanol, 99/1) to afford 15.56 g (75%) of **2** as a pale-yellow solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.72 (s, 1 H), 7.70 (s, 1 H), 7.50−7.48 (m, 1 H), 4.38 (q, J = 7.0 Hz, 2 H), 1.35 (t, J = 7.1 Hz, 3 H).

***N*-(4-Chlorobenzyl)-4-hydroxythieno[2,3-*b*]pyridine-5-carboxamide (3).** A mixture of ester **2** (74.8 g, 0.335 mol) and 4-chlorobenzylamine (237.2 g, 1.675 mol) was heated to 190 °C for 1 h with removal of ethanol via a Dean−Stark trap and then allowed to cool to 120 °C. A solution of toluene (800 mL) and ethanol (15 mL) was added, and the mixture was allowed to cool to room temperature. The resulting white precipitate was filtered, washed with toluene (200 mL), and recrystallized from acetic acid (800 mL)/water (800 mL) to yield 95.69 g (90%) of **3** as a white solid. Mp 238−240 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 13.4 (s, 1 H), 10.6 (br, 1 H), 8.72 (s, 1 H), 7.45−7.34 (m, 6 H), 4.55 (d, J = 5.9 Hz, 2 H); ^{13}C NMR (75 MHz, CF_3CO_2D) δ 169.0, 167.1, 153.6, 139.5, 134.7, 133.2, 128.8, 128.8, 127.8, 127.6, 107.9, 43.8; MS (ESI$^-$) m/z 317 (100, (M − H)$^-$). Anal. ($C_{15}H_{11}ClN_2O_2S$) C, H, N, Cl, S.

***N*-(4-Chlorobenzyl)-4-hydroxy-2-iodothieno[2,3-*b*]-pyridine-5-carboxamide (4).** Carboxamide **3** (7.30 g, 22.9 mmol) and sodium acetate (5.64 g, 68.7 mmol, 3.0 equiv) were dissolved in a mixture of chloroform/methanol (10/1, 440 mL). A solution of iodine monochloride (11.15 g, 68.7 mmol, 3.0 equiv) in methanol (10 mL) was then added to the mixture. The reaction mixture was stirred at room temperature for 1 h and then poured into a mixture of ice and saturated aqueous sodium bisulfite (400 mL). After being stirred vigorously for 30 min, the aqueous mixture was partially concentrated in vacuo to remove chloroform. The resulting aqueous suspension was filtered, washed with water (20 mL), and dried. The crude product was recrystallized by dissolving in methanol (1 L), and the resulting solution was filtered, concentrated in vacuo to ~400 mL, and cooled to 0 °C. Filtration afforded 6.33 g (62%) of **4** as white solid. Mp 230 °C (dec); ^{1}H NMR (300 MHz, DMSO-d_6) δ 13.40 (bs, 1 H), 10.40 (t, J = 5.9 Hz, 1 H), 8.68 (s, 1 H), 7.63 (s, 1 H), 7.41−7.33 (m, 4 H), 4.52 (d, J = 5.9 Hz, 2 H). Anal. ($C_{15}H_{10}ClIN_2O_2S$) C, H, N, Cl, S.

***N*-(4-Chlorobenzyl)-2-iodo-7-methyl-4-oxo-4,7-dihydrothieno[2,3-*b*]pyridine-5-carboxamide (5).** Potassium carbonate (1.76 g, 12.7 mmol, 3.0 equiv) and iodomethane (0.79 mL, 12.7 mmol, 3.0 equiv) were added to a solution of **4** (2.00 g, 4.23 mmol) in DMF (14 mL). The reaction mixture was heated to 90 °C and stirred for 18 h. The mixture was concentrated in vacuo. The resulting residue was partitioned between water (100 mL) and CH_2Cl_2 (200 mL). The aqueous layer was extracted with CH_2Cl_2 (3 × 150 mL). The combined organic layers were dried ($MgSO_4$), filtered, and concentrated in vacuo. The resulting solid was recrystallized from ethanol to yield 1.78 g (92%) of **5** as an off-white solid. Mp 236−237 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.45 (t, J = 5.9 Hz, 1 H), 8.69 (s, 1 H), 7.71 (s, 1 H), 7.41−7.32 (m, 4 H), 4.54 (d, J = 5.9 Hz, 2 H), 3.93 (s, 3 H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 171.1, 164.5, 154.8, 145.9, 139.0, 133.2, 132.5, 131.9, 129.6, 128.8, 115.0, 73.5, 43.5, 41.9; MS (ESI$^+$) m/z 459 ((M + H)$^+$, 100). Anal. ($C_{16}H_{12}ClIN_2O_2S$) C, H, N, Cl, S.

***N*-((4-Chlorophenyl)methyl)-4-hydroxy-2-(3-hydroxy-1-propynyl)-thieno[2,3-*b*]pyridine-5-carboxamide (6a).** Copper(I) iodide (0.128 g, 0.675 mmol, 0.3 equiv) and $PdCl_2(PPh_3)_2$ (0.032 g, 0.045 mmol, 0.02 equiv) were added to a suspension of **4** (1.00 g, 2.25 mmol) in diethylamine (28 mL). Propargyl alcohol (0.16 mL, 2.70 mmol, 1.2 equiv) was added, and the mixture was stirred at room temperature for 3 days. The reaction mixture was partitioned between water (100 mL) and ethyl acetate (100 mL), and the aqueous layer was extracted with ethyl acetate (2 × 100 mL). The combined organic layers were washed with a saturated aqueous ammonium chloride solution (100 mL), dried ($MgSO_4$), filtered, and concentrated in vacuo. The resulting solid was purified by column chromatography (CH_2Cl_2/methanol; 98/2, 95/5) followed by recrystallization from CH_2Cl_2/methanol to yield 0.165 g (20%) of **6a** as an off-white solid. Mp 233−236 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 13.32 (bs, 1 H), 10.41 (bs, 1 H), 8.75 (s, 1 H), 7.49 (s, 1 H), 7.41−7.33 (m, 4 H), 5.44 (bs, 1 H), 4.53 (d, J = 5.9 Hz, 2 H), 4.34 (s, 2 H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 172.8, 164.9, 148.8, 143.0, 131.9, 130.9, 129.6, 128.8, 126.8, 117.1, 114.2, 96.4, 76.6, 50.0, 41.9; MS (ESI$^-$) m/z 371 (100, (M − H)$^-$). Anal. ($C_{18}H_{13}ClN_2O_3S$) C, H, N, Cl, S.

***N*-((4-Chlorophenyl)methyl)-4,7-dihydro-7-ethyl-2-(3-hydroxy-1-propynyl)-4-oxo-thieno[2,3-*b*]pyridine-5-carboxamide (6b).** Potassium carbonate (0.149 g, 1.08 mmol, 2.0 equiv) and iodoethane (0.09 mL, 1.08 mmol, 2.0 equiv) were added to a solution of **6a** (0.200 g, 0.54 mmol) in DMF (10 mL). The mixture was stirred at room temperature for 3 days. The reaction mixture was poured into water (20 mL). The resulting solid was filtered and recrystallized from ethanol to yield 0.140 g (65%) of **6b** as an off-white solid. Mp 220−221 °C (dec); ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.42 (t, J = 5.9 Hz, 1 H), 8.79 (s, 1 H), 7.57 (s, 1 H), 7.41−7.33 (m, 4 H), 5.49 (br, 1 H), 4.55 (d, J = 5.9 Hz, 2 H), 4.36 (s, 2 H), 4.31 (q, J = 7.2 Hz, 2 H), 1.44 (t, J = 7.2 Hz, 3 H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.7, 163.8, 149.1, 144.9, 138.4, 131.3, 130.9, 129.0, 128.3, 127.4, 116.3, 115.0, 96.3, 75.6, 51.8, 49.4, 41.3, 13.6; MS (ESI$^+$) m/z 401 ((M + H)$^+$, 100). Anal. ($C_{20}H_{17}ClN_2O_3S$·0.1EtOH) C, H, N, Cl, S.

***N*-(4-Chlorobenzyl)-2-(3-hydroxy-1-propynyl)-7-isopropyl-4-oxo-4,7-dihydrothieno[2,3-*b*]pyridine-5-carboxamide (6c).** Potassium carbonate (0.298 g, 2.16 mmol, 2.0 equiv) and 2-bromopropane (0.20 mL, 2.16 mmol, 4.0 equiv) were added to a solution of **6a** (0.200 g, 0.54 mmol) in DMF

(10 mL). The mixture was heated to 60 °C for 18 h and then was partitioned between water (20 mL) and CH_2Cl_2 (25 mL). The aqueous layer was extracted with CH_2Cl_2 (3 × 25 mL). The combined organic layers were washed with brine (50 mL), dried ($MgSO_4$), filtered, and concentrated in vacuo. The resulting oil was purified by column chromatography (CH_2Cl_2/methanol, 99/1) followed by recrystallization from methanol to yield 0.043 g (19%) of **6c** as a yellow solid. Mp 191−197 °C (dec); ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.42 (t, J = 6.0 Hz, 1 H), 8.71 (s, 1 H), 7.58 (s, 1 H), 7.41−7.34 (m, 4 H), 5.48 (t, J = 6.0 Hz, 1 H), 4.55 (d, J = 5.8 Hz, 2 H), 4.55−4.48 (m, 1 H), 4.35 (s, 2 H), 1.57 (d, J = 6.6 Hz, 6 H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.6, 163.7, 149.1, 140.7, 138.4, 131.3, 131.1, 129.1, 128.2, 127.4, 116.3, 115.1, 96.3, 75.5, 59.0, 49.4, 41.4, 20.8; MS (ESI$^+$) m/z 415 ((M + H)$^+$, 100). Anal. ($C_{21}H_{19}ClN_2O_3S$) C, H, N, Cl, S.

***N*-(4-Chlorobenzyl)-7-(2-hydroxyethyl)-2-(3-hydroxy-1-propynyl)-4-oxo-4,7-dihydrothieno[2,3-*b*]pyridine-5-carboxamide (6d).** Potassium carbonate (0.739 g, 5.35 mmol, 5.0 equiv) and 2-bromoethanol (0.76 mL, 10.7 mmol, 10.0 equiv) were added to a solution of **6a** (0.400 g, 1.07 mmol) in DMF (20 mL). The reaction mixture was heated to 100 °C and stirred for 5 h. The mixture was partitioned between water (50 mL) and CH_2Cl_2 (50 mL). The aqueous layer was extracted with CH_2Cl_2 (3 × 50 mL). The combined organic layers were washed with brine (50 mL), dried ($MgSO_4$), filtered, and concentrated in vacuo. The resulting solid was purified by column chromatography (CH_2Cl_2/methanol; 99/1, 97/3, 96/4) followed by recrystallization from ethanol to yield 0.131 g (29%) of **6d** as an off-white solid. Mp 225−227 °C (dec); ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.43 (t, J = 6.0 Hz, 1 H), 8.70 (s, 1 H), 7.56 (s, 1 H), 7.41−7.33 (m, 4 H), 5.48 (t, J = 5.9 Hz, 1 H), 5.17 (t, J = 5.4 Hz, 1 H), 4.55 (d, J = 5.9 Hz, 2 H), 4.36 (d, J = 5.6 Hz, 2 H), 4.31 (t, J = 4.8 Hz, 2 H), 3.81−3.78 (m, 2 H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.8, 163.8, 149.9, 146.1, 138.4, 131.3, 130.8, 129.0, 128.3, 127.2, 116.1, 114.4, 96.2, 75.6, 59.2, 58.2, 49.4, 41.3; MS (ESI$^+$) m/z 417 ((M + H)$^+$, 100). Anal. ($C_{20}H_{17}ClN_2O_4S$) C, H, N, Cl, S.

***N*-(4-Chlorobenzyl)-7-(2-(diethylamino)ethyl)-2-(3-hydroxy-1-propynyl)-4-oxo-4,7-dihydrothieno[2,3-*b*]pyridine-5-carboxamide (6e).** Potassium carbonate (2.22 g, 16.0 mmol, 15.0 equiv) and 2-bromo-*N*,*N*-diethylethylamine hydrobromide (2.79 g, 10.7 mmol, 10.0 equiv) were added to a solution of **6a** (0.400 g, 1.07 mmol) in DMF (20 mL). The reaction mixture was heated to 100 °C and stirred for 18 h. The mixture was poured into water (40 mL). The resulting solid was filtered and purified by column chromatography (CH_2Cl_2/methanol, 98/2) followed by recrystallization from ethanol to yield 0.351 g (53%) of **6e** as a yellow solid. Mp 139−141 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.40 (t, J = 5.9 Hz, 1 H), 8.71 (s, 1 H), 7.55 (s, 1 H), 7.41−7.34 (m, 4 H), 5.47 (t, J = 6.0 Hz, 1 H), 4.54 (d, J = 5.9 Hz, 2 H), 4.36 (d, J = 6.0 Hz, 2 H), 4.29 (t, J = 5.4 Hz, 2 H), 2.77 (t, J = 5.5 Hz, 2 H), 2.46 (t, J = 7.0 Hz, 4 H), 0.78 (t, J = 7.0 Hz, 6 H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.7, 163.8, 149.7, 146.4, 138.3, 131.3, 130.7, 129.0, 128.3, 127.3, 116.0, 114.0, 96.2, 75.6, 55.2, 50.2, 49.4, 46.4, 41.4, 11.9; MS (ESI$^+$) m/z 472 ((M + H)$^+$, 100). Anal. ($C_{24}H_{26}ClN_3O_3S$) C, H, N, Cl, S.

***N*-(4-Chlorobenzyl)-4-hydroxy-2-(3-hydroxypropyl)-thieno[2,3-*b*]pyridine-5-carboxamide (7a).** Compound **6a** (0.300 g, 0.805 mmol) in CH_2Cl_2/methanol (1/1, 70 mL) was hydrogenated (35 psi) in the presence of 10% Pd/C (90 mg). The reaction mixture was filtered through a Celite pad, and the filtrate was concentrated in vacuo. The resulting solid was purified by column chromatography (CH_2Cl_2/methanol; 98/2, 95/5) followed by recrystallization from ethanol to yield 0.083 g (27%) of **7a** as a white, crystalline solid. Mp 185−186 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 13.29 (bs, 1 H), 10.61 (br, 1 H), 8.64 (s, 1 H), 7.42−7.33 (m, 4 H), 7.11 (s, 1 H), 4.54 (d, J = 5.9 Hz, 2 H), 4.53 (br, 1 H), 3.46−3.43 (m, 2 H), 2.87 (t, J = 7.4 Hz, 2 H), 1.83−1.74 (m, 2 H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 173.1, 165.2, 147.3, 141.3, 140.5, 139.1, 131.9, 129.6, 128.8, 118.7, 113.8, 60.0, 41.9, 34.3, 26.5; MS (ESI$^-$) m/z 376 ((M − H)$^-$, 100). Anal. ($C_{18}H_{17}ClN_2O_3S$) C, H, N, Cl, S.

***N*-(4-Chlorobenzyl)-7-ethyl-2-(3-hydroxypropyl)-4-oxo-4,7-dihydrothieno[2,3-*b*]pyridine-5-carboxamide (7b).** Compound **6b** (0.197 g, 0.491 mmol) in a mixture of CH_2Cl_2/methanol (1/1, 50 mL) was hydrogenated (35 psi) in the presence of 10% Pd/C (59 mg). The reaction mixture was filtered through a Celite pad, and the filtrate was concentrated in vacuo. The resulting solid was purified by column chromatography (CH_2Cl_2; CH_2Cl_2/methanol; 98/2, 95/5) to yield 0.114 g (57%) of **7b** as a white solid. Mp 144−146 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.62 (t, J = 5.9 Hz, 1 H), 8.72 (s, 1 H), 7.41−7.33 (m, 4 H), 7.20 (s, 1 H), 4.56 (t, J = 5.2 Hz, 1 H), 4.55 (d, J = 5.8 Hz, 2 H), 4.31 (q, J = 7.2 Hz, 2 H), 3.47 (q, J = 6.0 Hz, 2 H), 2.90 (t, J = 7.5 Hz, 2 H), 1.80 (qt, J = 7.5 Hz, 2 H), 1.44 (t, J = 7.2 Hz, 3 H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 172.3, 164.8, 148.6, 144.2, 140.5, 139.1, 132.1, 131.8, 129.6, 128.8, 120.0, 115.1, 60.0, 52.1, 41.9, 34.3, 26.5, 14.4; MS (ESI$^-$) m/z 403 ((M − H)$^-$, 100). Anal. ($C_{20}H_{21}ClN_2O_3S$) C, H, N, Cl, S.

***N*-(4-Chlorobenzyl)-2-(3-hydroxypropyl)-7-isopropyl-4-oxo-4,7-dihydrothieno[2,3-*b*]pyridine-5-carboxamide (7c).** Compound **6c** (0.225 g, 0.542 mmol) in ethanol (50 mL) was hydrogenated (35 psi) in the presence of 10% Pd/C (68 mg). The reaction mixture was filtered through a Celite pad, and the filtrate was concentrated in vacuo. The resulting solid was recrystallized from ethyl acetate/heptane to yield 0.104 g (46%) of **7c** as an off-white solid. Mp 84−92 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.61 (t, J = 5.9 Hz, 1 H), 8.66 (s, 1 H), 7.41−7.33 (m, 4 H), 7.21 (s, 1 H), 4.59−4.50 (m, 4 H), 3.47 (q, J = 6.2 Hz, 2 H), 2.91 (t, J = 7.5 Hz, 2 H), 1.80 (qt, J = 7.6 Hz, 2 H), 1.57 (d, J = 6.6 Hz, 6 H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 172.2, 164.8, 148.6, 140.4, 140.0, 139.1, 132.2, 131.9, 129.7, 128.8, 120.0, 115.1, 60.0, 59.0, 41.9, 34.3, 26.4, 21.5; MS (FAB) m/z 419 (MH$^+$, 99); HRMS (FAB) calcd for $C_{21}H_{23}ClN_2O_3S$ + H (M + H)$^+$ m/z 419.1196, found 419.1172. Anal. ($C_{21}H_{23}ClN_2O_3S$) C, H, N, Cl, S.

***N*-(4-Chlorobenzyl)-7-(2-hydroxyethyl)-2-(3-hydroxypropyl)-4-oxo-4,7-dihydrothieno[2,3-*b*]pyridine-5-carboxamide (7d).** Compound **6d** (0.200 g, 0.48 mmol) in THF (90 mL) was hydrogenated (35 psi) in the presence of 10% Pd/C (60 mg). The reaction mixture was filtered through a Celite pad, and the filtrate was concentrated in vacuo. The resulting solid was purified by column chromatography (CH_2Cl_2/methanol, 97/3) followed by recrystallization from acetonitrile to yield 0.066 g (33%) of **7d** as a pale-yellow solid. Mp 185−191 °C (dec); ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.62 (t, J = 5.9 Hz, 1 H), 8.63 (s, 1 H), 7.41−7.33 (m, 4 H), 7.19 (s, 1 H), 5.15 (t, J = 5.4 Hz, 1 H), 4.58 (t, J = 5.1 Hz, 1 H), 4.55 (d, J = 6.0 Hz, 2 H), 4.29 (t, J = 4.8 Hz, 2 H), 3.81−3.77 (m, 2 H), 3.48−3.44 (m, 2 H), 2.90 (t, J = 7.5 Hz, 2 H), 1.83−1.76 (m, 2 H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.8, 164.3, 148.6, 144.9, 139.7, 138.5, 131.4, 131.3, 129.0, 128.2, 119.3, 113.8, 59.4, 58.8, 58.1, 41.3, 33.7, 25.8; MS (ESI$^+$) m/z 421 ((M + H)$^+$, 100). Anal. ($C_{20}H_{21}ClN_2O_4S$) C, H, N, Cl, S.

***N*-(4-Chlorobenzyl)-7-(2-(diethylamino)ethyl)-2-(3-hydroxypropyl)-4-oxo-4,7-dihydrothieno[2,3-*b*]pyridine-5-carboxamide (7e).** Compound **6e** (0.200 g, 0.42 mmol) in THF (80 mL) was hydrogenated (35 psi) in the presence of 10% Pd/C (60 mg). The reaction mixture was filtered through a Celite pad, and the filtrate was concentrated in vacuo. The resulting oil was purified by column chromatography (CH_2Cl_2/methanol; 98/2, 96/4) to yield 0.123 g (62%) of **7e** as a pale-yellow solid. Mp 78−82 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.59 (t, J = 5.9 Hz, 1 H), 8.63 (s, 1 H), 7.41−7.33 (m, 4 H), 7.18 (s, 1 H), 4.57 (t, J = 5.1 Hz, 1 H), 4.54 (d, J = 5.9 Hz, 2 H), 4.27 (t, J = 5.6 Hz, 2 H), 3.47−3.43 (m, 2 H), 2.90 (t, J = 7.4 Hz, 2 H), 2.77 (t, J = 5.7 Hz, 2 H), 2.46 (q, J = 7.0 Hz, 4 H), 1.83−1.76 (m, 2 H), 0.78 (t, J = 7.0 Hz, 6 H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.7, 164.3, 148.4, 145.2, 139.6, 138.5, 131.3, 129.0, 128.2, 119.4, 113.5, 59.3, 54.9, 50.3, 46.4, 43.2, 41.3, 33.7, 25.9, 11.9; MS (ESI$^+$) m/z 476 ((M + H)$^+$, 100). Anal. ($C_{24}H_{30}ClN_3O_3S$) C, H, N, Cl, S.

Methyl 5-(((4-Chlorobenzyl)amino)carbonyl)-4-hydroxythieno[2,3-*b*]pyridine-2-carboxylate (8). Triethylamine (15.6 mL, 112 mmol, 2.0 equiv) and methanol (91 mL, 2.25 mol, 40

equiv) were added to a solution of **4** (25.0 g, 56.2 mmol) in DMF (400 mL). Nitrogen was bubbled through the solution for 15 min to degas the reaction mixture. $PdCl_2(PPh_3)_2$ (7.86 g, 11.2 mmol, 0.20 equiv) was added. Carbon monoxide was bubbled through the solution. The reaction mixture was stirred at 70 °C with continuous carbon monoxide addition for 18 h. The mixture was allowed to cool to room temperature and was poured into ice/water (2000 mL). The resulting mixture was filtered through Celite. The filtrate was divided into two equal portions, and each was extracted with CH_2Cl_2 (4 × 500 mL). The combined organic layers were dried ($MgSO_4$), filtered, and concentrated in vacuo. The resulting solid was recrystallized from methanol to yield 14.65 g (69%) of **8** as a white, crystalline solid. Mp 238−240 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 13.45 (br, 1 H), 10.30 (t, J = 5.8 Hz, 1 H), 8.80 (s, 1 H), 7.96 (s, 1 H), 7.41−7.33 (m, 4 H), 4.55 (d, J = 6.0 Hz, 2 H), 3.87 (s, 3 H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 173.3, 164.1, 161.7, 151.4, 143.5, 138.5, 131.4, 130.6, 129.1, 128.3, 127.9, 126.4, 114.0, 52.7, 41.4; MS (ESI$^-$) m/z 375 (100, (M − H)$^-$). Anal. ($C_{17}H_{13}ClN_2O_4S\cdot 0.9H_2O$) C, H, N, Cl, S.

***N*-(4-Chlorobenzyl)-4-hydroxy-2-(hydroxymethyl)-thieno[2,3-*b*]pyridine-5-carboxamide (9).** Ester **8** (0.506 g, 1.34 mmol) was dissolved in THF (100 mL) with heating, and then the reaction mixture was cooled in an ice bath. To this solution was added a solution of lithium aluminum hydride (1.0 M in THF, 2.4 mL, 2.4 mmol, 1.8 equiv). The reaction mixture was allowed to warm to room temperature and was stirred for 2.5 h. The reaction was quenched with water (1 mL), 10% NaOH (1 mL), and water (1 mL). The aluminum salts were removed by filtration, and the filtrate was concentrated in vacuo. The resulting oil was purified by column chromatography (CH_2Cl_2/methanol; 98/2, 95/5) to yield 0.254 g (54%) of **9** as a pale-yellow solid. Mp 205−210 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 13.34 (br, 1 H), 10.57 (br, 1 H), 8.66 (s, 1 H), 7.41−7.33 (m, 4 H), 7.22 (s, 1 H), 5.69 (br, 1 H), 4.68 (br, 2 H), 4.54 (d, J = 5.9 Hz, 2 H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 172.4, 164.9, 148.5, 141.8, 141.4, 138.6, 131.4, 130.5, 129.2, 128.3, 117.3, 113.2, 58.5, 41.4; MS (ESI$^-$) for m/z 347 (100, (M − H)$^-$). Anal. ($C_{16}H_{13}ClN_2O_3S$) C, H, N, Cl, S.

***N*-(4-Chlorobenzyl)-2-(hydroxymethyl)-7-methyl-4-oxo-4,7-dihydrothieno[2,3-*b*]pyridine-5-carboxamide (10a).** Potassium carbonate (0.567 g, 4.10 mmol, 1.5 equiv) and iodomethane (0.20 mL, 3.28 mmol, 1.2 equiv) were added to a solution of **9** (0.955 g, 2.73 mmol) in DMF (20 mL). The reaction mixture was stirred at room temperature for 1 h. The mixture was partitioned between water (50 mL) and CH_2Cl_2 (100 mL). The organic layer was removed in vacuo, and the resulting precipitate was filtered and recrystallized from ethanol to yield 0.825 g (83%) of **10a** as a white, crystalline solid. Mp 222−225 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.59 (t, J = 5.9 Hz, 1 H), 8.70 (s, 1 H), 7.41−7.33 (m, 4 H), 7.30 (s, 1 H), 5.79 (t, J = 5.7 Hz, 1 H), 4.72 (d, J = 5.5 Hz, 2 H), 4.55 (d, J = 5.9 Hz, 2 H), 3.96 (s, 3 H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 172.0, 164.4, 150.1, 145.2, 141.9, 138.6, 131.4, 130.7, 129.1, 128.3, 118.4, 114.3, 58.4, 42.9, 41.4; MS (ESI$^+$) m/z 363 (100, (M + H)$^+$). Anal. ($C_{17}H_{15}ClN_2O_3S$) C, H, N, Cl, S.

***N*-(4-Chlorobenzyl)-7-methyl-4-oxo-2-vinyl-4,7-dihydrothieno[2,3-*b*]pyridine-5-carboxamide (11).** $PdCl_2(PPh_3)_2$ (0.107 g, 0.153 mmol, 0.02 equiv) and tributylvinylstannane (2.34 mL, 8.01 mmol, 1.05 equiv) were added to a suspension of **5** (3.50 g, 7.63 mmol) in DMF (110 mL). The reaction mixture was stirred at 45 °C for 18 h. The mixture was allowed to cool to room temperature, was poured into water (200 mL), and was extracted with CH_2Cl_2 (3 × 200 mL). The combined organic layers were washed with water (50 mL) and brine (2 × 50 mL), dried ($MgSO_4$), filtered, and concentrated in vacuo. The resulting solid was purified by column chromatography (CH_2Cl_2/methanol, 99/1) followed by recrystallization from acetonitrile to yield 2.372 g (87%) of **11** as a pale-yellow, crystalline solid. Mp 205−210 °C (dec); ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.52 (t, J = 5.9 Hz, 1 H), 8.71 (s, 1 H), 7.46 (s, 1 H), 7.41−7.33 (m, 4 H), 7.04−6.95 (m, 1 H), 5.65 (d, J = 17.3 Hz, 1 H), 5.35 (d, J = 10.9 Hz, 1 H), 4.56 (d, J = 5.9 Hz, 2 H), 3.96 (s, 3 H); ^{13}C NMR (DMSO-d_6) δ 171.8, 164.1, 149.6, 145.6, 138.5, 136.6, 131.3, 131.1, 129.7, 129.0, 128.2, 121.8, 115.7, 114.6, 42.9, 41.3; MS (ESI$^+$) m/z 359 (100, (M + H)$^+$); HRMS (FAB) calcd for $C_{18}H_{15}ClN_2O_2S$ + H m/z 359.0621, found 359.0621. Anal. ($C_{18}H_{15}ClN_2O_2S$) C (calcd 60.25, found 59.28), H, N, Cl, S.

***N*-(4-Chlorobenzyl)-2-(2-hydroxyethyl)-7-methyl-4-oxo-4,7-dihydrothieno[2,3-*b*]pyridine-5-carboxamide (10b).** Carboxamide **11** (1.40 g, 3.90 mmol) was dissolved in THF (280 mL) with heating and then allowed to cool to room temperature. A solution of 9-BBN (23.4 mL, 0.5 M in THF, 11.7 mmol, 3.0 equiv) was added. The reaction mixture was stirred at room temperature for 2 h, and an additional 15.6 mL of 9-BBN was then added. The reaction mixture was stirred at room temperature for 18 h, and 2 N NaOH (140 mL) followed by 30% hydrogen peroxide (140 mL) were added. The mixture was stirred at room temperature for 1 h and was then extracted with CH_2Cl_2 (3 × 300 mL). The combined organic layers were washed with brine (150 mL), dried ($MgSO_4$), filtered, and concentrated in vacuo. The resulting oil was purified by column chromatography (CH_2Cl_2/methanol; 99/1, 98/2, 97/3) followed by sequential recrystallization from acetonitrile and ethanol to yield 0.155 g (11%) of **10b** as a white solid. Mp 213-214 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.63 (t, J = 5.9 Hz, 1 H), 8.70 (s, 1 H), 7.42−7.33 (m, 4 H), 7.25 (s, 1 H), 4.99 (t, J = 5.1 Hz, 1 H), 4.55 (d, J = 5.9 Hz, 2 H), 3.95 (s, 3 H), 3.67 (q, J = 6.0 Hz, 2 H), 3.00 (t, J = 6.0 Hz, 2 H); ^{13}C NMR (DMSO-d_6) δ 171.7, 164.4, 149.8, 144.9, 138.5, 137.4, 131.3, 130.6, 129.0, 128.2, 120.1, 114.0, 60.9, 42.7, 41.2, 33.1; MS (ESI$^+$) m/z 377 (100, (M + H)$^+$); HRMS (FAB) calcd for $C_{18}H_{17}ClN_2O_3S$ +H m/z 377.0726, found 377.0723. Anal. ($C_{18}H_{17}ClN_2O_3S\cdot 1.0H_2O$) C, H, N, Cl, S.

***N*-(4-Chlorobenzyl)-2-(3-hydroxy-1-propynyl)-7-methyl-4-oxo-4,7-dihydrothieno[2,3-*b*]pyridine-5-carboxamide (12a).** Copper(I) iodide (0.210 g, 1.07 mmol, 0.30 equiv), **5** (1.632 g, 3.56 mmol), and $Pd(PPh_3)_2Cl_2$ (0.125 g, 0.178 mmol, 0.05 equiv) were suspended in diethylamine (40 mL). Propargyl alcohol (0.29 mL, 4.98 mmol, 1.4 equiv) was added, and the reaction mixture was stirred at room temperature for 18 h. Diethylamine was removed in vacuo and the resulting solid was purified by column chromatography (CH_2Cl_2/methanol, 98/2) followed by recrystallization from ethanol to yield 1.06 g (77%) of **12a** as a yellow solid. Mp 206−208 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.42 (t, J = 5.8 Hz, 1 H), 8.76 (s, 1 H), 7.57 (s, 1 H), 7.41−7.33 (m, 4 H), 5.47 (t, J = 6.0 Hz, 1 H), 4.55 (d, J = 5.9 Hz, 2 H), 4.37 (d, J = 6.0 Hz, 2 H), 3.95 (s, 3 H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 172.2, 164.4, 151.0, 146.8, 138.9, 131.9, 130.9, 129.6, 128.8, 128.0, 116.9, 115.2, 96.8, 76.2, 50.0, 43.6, 41.9; MS (FAB) m/z 387 (MH$^+$, 99); HRMS (FAB) calcd for $C_{19}H_{15}ClN_2O_3S$ + H (M + H)$^+$ m/z 387.0570, found 387.0558. Anal. ($C_{19}H_{15}ClN_2O_3S\cdot 0.3H_2O$) C, H, N, Cl, S.

***N*-(4-Chlorobenzyl)-2-(4-hydroxy-1-butynyl)-7-methyl-4-oxo-4,7-dihydrothieno[2,3-*b*]pyridine-5-carboxamide (12b).** Copper(I) iodide (0.125 g, 0.654 mmol, 0.30 equiv), **5** (1.00 g, 2.18 mmol), and $Pd(PPh_3)_2Cl_2$ (0.077 g, 0.109 mmol, 0.05 equiv) were suspended in diethylamine (25 mL). 3-Butyn-1-ol (0.23 mL, 3.05 mmol, 1.4 equiv) was added, and the reaction mixture was stirred at room temperature for 18 h. Diethylamine was removed in vacuo and the resulting brown solid was purified by column chromatography (CH_2Cl_2; CH_2Cl_2/methanol, 99/1) to provide a yellow solid that was recrystallized from methanol to yield 0.668 g (76%) of **12b** as an off-white solid. Mp 199−206 °C (dec); ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.44 (t, J = 5.9 Hz, 1 H), 8.75 (s, 1 H), 7.49 (s, 1 H), 7.41−7.33 (m, 4 H), 4.98 (t, J = 5.0 Hz, 1 H), 4.54 (d, J = 5.9 Hz, 2 H), 3.94 (s, 3 H), 3.61−3.57 (m, 2 H), 2.63 (t, J = 6.6 Hz, 2 H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.6, 163.9, 149.9, 146.0, 138.4, 131.3, 130.3, 129.0, 128.2, 126.5, 117.4, 114.5, 95.7, 73.0, 59.2, 43.0, 41.3, 23.5; MS (ESI$^+$) m/z 401 (100, (M + H)$^+$). Anal. ($C_{20}H_{17}ClN_2O_3S\cdot 0.5H_2O$): C, H, N, Cl, S.

***N*-(4-Chlorobenzyl)-2-(5-hydroxy-1-pentynyl)-7-methyl-4-oxo-4,7-dihydrothieno[2,3-*b*]pyridine-5-carboxamide (12c).** Copper(I) iodide (0.114 g, 0.600 mmol, 0.30 equiv), **5** (0.917 g, 2.00 mmol), and $Pd(PPh_3)_2Cl_2$ (0.070 g, 0.100 mmol, 0.05 equiv) were suspended in diethylamine (33 mL). 4-Pentyn-

1-ol (0.26 mL, 2.80 mmol, 1.4 equiv) was added, and the reaction mixture was stirred at room temperature for 18 h. Diethylamine was removed in vacuo, and the resulting solid was purified by column chromatography (CH_2Cl_2/methanol, 99/1) followed by recrystallization from acetonitrile to yield 0.693 g (84%) of **12c** as a pale-yellow solid. Mp 213-217 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.45 (t, J = 5.9 Hz, 1 H), 8.75 (s, 1 H), 7.48 (s, 1 H), 7.41−7.33 (m, 4 H), 4.58 (t, J = 5.1 Hz, 1 H), 4.54 (d, J = 6.0 Hz, 2 H), 3.94 (s, 3 H), 3.52−3.48 (m, 2 H), 2.56−2.51 (m, 2 H), 1.73−1.66 (m, 2 H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.6, 163.9, 149.8, 146.0, 138.4, 131.3, 130.4, 129.0, 128.2, 126.4, 117.4, 114.5, 97.5, 72.4, 59.2, 43.0, 41.3, 31.0, 15.6; MS (ESI$^+$) m/z 415 (100, (M + H)$^+$). Anal. ($C_{21}H_{19}ClN_2O_3S$) C, H, N, Cl, S.

***N*-(4-Chlorobenzyl)-2-(3-hydroxypropyl)-7-methyl-4-oxo-4,7-dihydrothieno[2,3-*b*]pyridine-5-carboxamide (10c).** Compound **12a** (0.520 g, 1.34 mmol) in CH_2Cl_2/ethanol (1/1, 160 mL) was hydrogenated (35 psi) in the presence of 10% Pd/C (0.156 g). The mixture was filtered through a Celite pad, and the filtrate was concentrated in vacuo. The resulting solid was purified by column chromatography (CH_2Cl_2/methanol, 98/2) followed by recrystallization from ethanol to yield 0.211 g (40%) of **10c** as an off-white solid. Mp 197−198 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.61 (t, J = 5.9 Hz, 1 H), 8.69 (s, 1 H), 7.41−7.33 (m, 4 H), 7.21 (s, 1 H), 4.58−4.53 (m, 3 H), 3.95 (s, 3 H), 3.47 (q, J = 6.1 Hz, 2 H), 2.91 (t, J = 7.5 Hz, 2 H), 1.80 (qt, J = 7.4 Hz, 2 H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 172.2, 164.9, 149.8, 145.5, 140.6, 139.1, 131.8, 131.5, 129.6, 128.8, 120.0, 114.7, 60.0, 43.4, 41.9, 34.4, 26.5; MS (ESI$^+$) m/z 391 (100, (M + H)$^+$); HRMS (FAB) calcd for $C_{19}H_{19}ClN_2O_3S$ + H (M + H)$^+$ m/z 391.0883, found 391.0904. Anal. ($C_{19}H_{19}ClN_2O_3S$) C (calcd 58.38, found 57.42), H, N, Cl, S.

***N*-(4-Chlorobenzyl)-2-(4-hydroxybutyl)-7-methyl-4-oxo-4,7-dihydrothieno[2,3-*b*]pyridine-5-carboxamide (10d).** Compound **12b** (0.400 g, 1.00 mmol) in CH_2Cl_2/ethanol (1/1, 120 mL) was hydrogenated (35 psi) in the presence of 10% Pd/C (0.120 g). The reaction mixture was filtered through a Celite pad, and the filtrate was concentrated in vacuo. The resulting solid was purified by column chromatography (CH_2Cl_2/methanol; 99/1, 98/2) followed by recrystallization from methanol to yield 0.279 g (69%) of **10d** as a white solid. Mp 186−188 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.62 (t, J = 6.0 Hz, 1 H), 8.70 (s, 1 H), 7.41−7.33 (m, 4 H), 7.21 (s, 1 H), 4.55 (d, J = 5.9 Hz, 2 H), 4.39 (br, 1 H), 3.95 (s, 3 H), 3.42 (t, J = 5.8 Hz, 2 H), 2.89 (t, J = 7.3 Hz, 2 H), 1.72−1.64 (m, 2 H), 1.51−1.44 (m, 2 H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.7, 164.3, 149.2, 144.9, 140.3, 138.5, 131.3, 130.9, 129.0, 128.2, 119.4, 114.1, 60.1, 42.8, 41.3, 31.5, 29.0, 27.2; MS (ESI$^+$) m/z 405 (100, (M + H)$^+$). Anal. ($C_{20}H_{21}ClN_2O_3S$·1.0 H_2O) C, H, N, Cl, S.

***N*-(4-Chlorobenzyl)-2-(5-hydroxypentyl)-7-methyl-4-oxo-4,7-dihydrothieno[2,3-*b*]pyridine-5-carboxamide (10e).** Compound **12c** (0.400 g, 0.964 mmol) in CH_2Cl_2/ethanol (1/1, 140 mL) was hydrogenated (35 psi) in the presence of 10% Pd/C (0.120 g). The reaction mixture was filtered through a Celite pad, and the filtrate was concentrated in vacuo. The resulting solid was purified by column chromatography (CH_2Cl_2/methanol; 99/1, 98/2) followed by recrystallization from ethanol to yield 0.251 g (62%) of **10e** as a white solid. Mp 160−164 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.62 (t, J = 5.9 Hz, 1 H), 8.70 (s, 1 H), 7.41−7.31 (m, 4 H), 7.21 (s, 1 H), 4.55 (d, J = 5.9 Hz, 2 H), 4.37 (t, J = 5.1 Hz, 1 H), 3.40−3.36 (m, 2 H), 2.87 (t, J = 7.3 Hz, 2 H), 1.69−1.62 (m, 2 H), 1.48−1.42 (m, 2 H), 1.39−1.33 (m, 2 H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.7, 164.3, 149.2, 144.9, 140.2, 138.5, 131.3, 130.9, 129.0, 128.2, 119.4, 114.1, 60.4, 42.8, 41.3, 32.0, 30.5, 29.3, 24.8; MS (ESI$^+$) m/z 419 (100, (M + H)$^+$). Anal. ($C_{21}H_{23}ClN_2O_3S$) C, H, N, Cl, S.

***N*-(4-Chlorobenzyl)-4-hydroxy-2-(4-morpholinylmethyl)thieno[2,3-*b*]pyridine-5-carboxamide (13).** An aqueous formaldehyde solution (37%, 2.6 mL, 34.5 mmol, 11 equiv) was added to morpholine (2.7 mL, 31.4 mmol, 10 equiv) at 0 °C. Ethanol (10 mL) was then added followed by addition of **3** (1.00 g, 3.14 mmol). Acetic acid (2 mL) was added, and the reaction mixture was allowed to warm to room temperature and then was refluxed for 18 h. Additional morpholine (2.7 mL, 31.4 mmol, 10 equiv) and formaldehyde (2.6 mL, 34.5 mmol, 11 equiv) were added, and the reaction mixture was refluxed for an additional 24 h. The reaction mixture was allowed to cool to room temperature and was then concentrated in vacuo. The residue was treated with 25% NaOH (20 mL). The aqueous layer was extracted with ethyl acetate (50 mL) and then with $CHCl_3$ (60 mL). Methanol (30 mL) was added to the aqueous layer, and it was extracted with $CHCl_3$ (2 × 60 mL). This procedure was repeated three times. The combined organic layers were dried ($MgSO_4$), filtered, and concentrated in vacuo. The resulting solid was purified by column chromatography (CH_2Cl_2/methanol; 98/2, 96/4) followed by recrystallization from ethyl acetate/Et_2O to yield 0.718 g (55%) of **13** as an off-white solid. Mp 193−195 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 13.30 (br, 1 H), 10.59 (t, J = 5.3 Hz, 1 H), 8.66 (s, 1 H), 7.41−7.33 (m, 4 H), 7.27 (s, 1 H), 4.54 (d, J = 5.9 Hz, 2 H), 3.72 (s, 2 H), 3.58 (t, J = 4.4 Hz, 4 H), 2.44 (t, J = 3.6 Hz, 4 H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 172.2, 164.8, 148.9, 141.4, 138.5, 137.5, 131.3, 130.3, 129.0, 128.2, 119.5, 113.1, 66.1, 56.7, 52.8, 41.2; MS (FAB) m/z 418 (MH$^+$, 99); HRMS (FAB) calcd for $C_{20}H_{20}ClN_3O_3S$ + H m/z 418.0992, found 418.0996. Anal. ($C_{20}H_{20}ClN_3O_3S$) C, H, N, Cl, S.

***N*-(4-Chlorobenzyl)-7-methyl-2-(4-morpholinylmethyl)-4-oxo-4,7-dihydrothieno[2,3-*b*]pyridine-5-carboxamide (14).** Potassium carbonate (6.50 g, 47.0 mmol, 2.0 equiv) and iodomethane (1.76 mL, 28.2 mmol, 1.2 equiv) were added to a solution of **13** (9.80 g, 23.5 mmol) in DMF (150 mL). The reaction mixture was stirred at room temperature for 18 h. The mixture was poured into water (300 mL). The resulting precipitate was filtered and recrystallized from acetonitrile to yield 7.90 g (78%) of **14** as a pale-yellow, crystalline solid. Mp 231−234 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.59 (t, J = 5.9 Hz, 1 H), 8.70 (s, 1 H), 7.41−7.33 (m, 5 H), 4.55 (d, J = 5.9 Hz, 2 H), 3.96 (s, 3 H), 3.75 (s, 2 H), 3.59 (t, J = 4.4 Hz, 4 H), 2.45 (br, 4 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.4, 164.4, 150.0, 143.9, 137.5, 136.8, 132.1, 131.0, 128.3, 128.0, 120.7, 115.1, 66.3, 57.1, 52.9, 42.5, 41.9; MS (ESI$^+$) m/z 432 ((M + H)$^+$, 100). Anal. ($C_{21}H_{22}ClN_3O_3S$) C, H, N, Cl, S.

DNA Polymerase Assay. HSV-1, HCMV, VZV, human α, and human δ DNA polymerases were expressed as C-terminal histidine-tagged proteins using a baculovirus expression system and purified by standard Ni affinity chromatography[16] (see Supporting Information). Plasmid constructs for HCMV (pwB747), HSV-1 (pDP4), and VZV (puC18) were obtained from D. Coen (Harvard University). Human α and δ DNA polymerases were cloned using standard techniques.[17] Purified human γ DNA polymerase was supplied by William Copeland (National Institutes of Health). Compounds were evaluated against the purified polymerases using a scintillation proximity assay (SPA). All compounds were solubilized in dimethyl sulfoxide (DMSO). Assay conditions for HSV-1, HCMV, VZV, human α, and human γ DNA polymerases were as follows: 5 μL of compound solution was added to 95 μL of polymerase in a solution containing 6.4 mM HEPES (pH 7.5), 12 mM KCl, 25 mM NaCl, 5 mM $MgCl_2$, 46 μg/mL of bovine serum albumin (BSA), 2 mM CHAPS, 5 mM dithiothreitol, 5% glycerol, 1.2 μCi of [*methyl*,1′,2′-^{3}H]TTP (Amersham Pharmacia Biotech), and 6 nM biotinylated oligo(dT)16-poly(dA) (Amersham Pharmacia Biotech). The polymerase reaction was incubated in a 96-well Dynatech Microlite I plate for 12 min at 26 °C and stopped by the addition of 50 μL of a 0.5 M EDTA (pH 8.0) solution containing 4 mg/mL SPA beads. The plate was sealed and counted on a Topcount microplate scintillation counter (Packard Instruments, Meriden, CT). Percent inhibition was determined for each drug concentration compared to the value of the uninhibited control. IC_{50} values were calculated using EXCEL software for linear regression. For human δ polymerase, 5 μL of compound solution was added to 95 μL of polymerase in a solution containing 20 mM HEPES (pH 7.5), 40 μg/mL of BSA, 2 mM $MnCl_2$, 1 mM dithiothreitol, 5% glycerol, 5% DMSO, 0.2 μM [*methyl*,1′,2′-^{3}H]TTP (Amersham Pharmacia Biotech), 0.4 μM dATP (Sigma, St. Louis, Mo.), and 10 μg/mL of poly(dA-dT) primer template. Reactions were

performed at 27 °C for 10 min. Incorporated TMP was precipitated with an equal volume of 10% trichloroacetic acid and collected on a GF/B Millipore multiscreen filtration plate. Wells were washed three times with 5% trichloroacetic acid and dried prior to the addition of 100 μL of Microscint 40 (Packard Instruments) per well. Zidovudine triphosphate (AZT-TP) (Moravek Biochemicals), phosphonoformic acid (foscarnet) (Sigma), and aphidicolin (Biomol) were used as standards in the DNA polymerase assay.

Plaque Reduction Assays. Antiviral activities of compounds against herpesviruses were determined using plaque reduction assays. HCMV (Davis strain) and VZV (Webster strain) were grown on human foreskin fibroblast (HFF) cells. HSV-1 (KOS strain) was grown on African green monkey kidney cells (Vero). HCMV Ad169 (V823A), HSV-1 KOS (V823A), and HSV-2 (V826A) were isolated as previously described.[14] Acyclovir-resistant HSV-1 and HSV-2 strains are denoted as follows with the coresponding location in polymerase gene in parentheses: AraA^{r}13 (V813M), PAA^{r}5 (R842S), PFA^{r}2 (R605V), and BW^r (N815S). They were obtained from D. Coen (Harvard University). The specific point mutations in the DNA polymerase gene resulting in resistance for these isolates have been described previously.[6,15] Approximately 50 PFU of virus was added to each well of a 24-well culture dish containing the appropriate cells. After a 1 h incubation at 37 °C, the virus inoculum was removed and drug-containing media was added. All compounds were solubilized in dimethyl sulfoxide (DMSO) as 200-fold stock solutions. Dilutions of compounds were then added to DMEM containing 10% FBS and 0.8% carboxymethyl cellulose. Acyclovir (Sigma) or ganciclovir (obtained from a wholesale pharmacy) was used as positive controls. The plates were incubated at 37 °C until plaques formed. Plaques were counted microscopically or by staining cells with crystal violet (0.1% in 20% ethanol). Percent inhibition compared to the value for the uninhibited control was determined for each drug concentration, and IC_{50} values were calculated using EXCEL software for linear regression.

Cell Viability Determinations. The toxicity of compounds on noninfected mammalian cells was determined for HFF and Vero cells seeded as subconfluent monolayers and treated with compound for 3 days. Cell viability determinations were performed using both microscopic evaluation and a quantitative neutral red dye uptake assay as previously described.[18]

Enzyme Kinetics Analysis for Compound 14. The K_i value of compound **14** for inhibition of [^{3}H]TTP incorporation into primer template with HCMV polymerase was determined by assessing various substrate and inhibitor concentrations in the standard in vitro polymerase assay. Substrate (TTP) concentrations spanned the calculated K_m for HCMV polymerase (1.2 μM), ranging from 0.25 to 8.0 μM. Data for dTTP incorporation was globally analyzed by the competitive inhibitor equation,

$$v = \frac{V_{max}S}{K_m\left(1 + \frac{I}{K_i}\right) + S} \quad (1)$$

using nonlinear regression analysis to yield a competitive K_i of 1.0 ± 0.1 μM.

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Supporting Information Available: Elemental analyses for compounds **1** and **3**−**14** and procedures for the expression and purification of HCMV polymerase. This material is available free of charge via the Internet at http://pubs.acs.org.

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