Acyclic Nucleoside Analogues as Novel Inhibitors of Human Mitochondrial Thymidine Kinase

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A series of acyclic nucleoside analogues of 5′-*O*-tritylthymidine have been synthesized and evaluated as potential human mitochondrial thymidine kinase (TK-2) inhibitors. In this series, the sugar moiety of the parent 5′-*O*-tritylthymidine has been replaced by aliphatic chains including (*E*)- and (*Z*)-butenol, butynol, or butanol. Among them the (*Z*)-butenyl derivative (**10**) showed an IC_{50} against TK-2 of 1.5 μM, being 1 order of magnitude more potent than the parent 5′-*O*-tritylthymidine. This lead compound has been further modified by replacing the thymine base by other pyrimidine bases such as 5-iodouracil, 5-ethyluracil, 5-methylcytosine, 3-*N*-methylthymine, or 5,6-dihydrothymine, as well as by the purine base guanine. The trityl group has also been replaced by different aliphatic and aromatic acyl moieties including *tert*-butylacetyl, hexanoyl, decanoyl, and diphenylacetyl moieties. The evaluation of the compounds against TK-2 and the phylogenetically close HSV-1 TK has shown that the base moiety plays a crucial role in their interaction against these pyrimidine nucleoside kinases. Also, the presence of a lipophilic substituent, preferentially an aromatic moiety such as diphenylmethyl or triphenylmethyl, is required for efficient TK-2 inhibition. Whereas some compounds showed marked specificity for either TK-2 (i.e, the 5,6-dihydrothymine derivative, **26**) or HSV-1 TK (i.e., the butynyl derivative, **11**), some others, including the (*Z*)-and (*E*)-butenyl derivatives **10** and **12**, showed significant inhibition against both enzymes. They also proved to be inhibitory against HSV-1 TK in intact human osteosarcoma cells that were transduced with the HSV-1 TK gene.

Introduction

In mammalian cells, there are two different nucleoside kinases that are able to phosphorylate thymidine to its 5′-monophosphate derivative, dTMP, namely, cytosolic thymidine kinase (TK-1) and mitochondrial thymidine kinase (TK-2).[1] Both enzymes show important differences in their primary amino acid sequence, substrate specificity, and level of expression in the different phases of the cell cycle. TK-1 reaches its peak of expression in the early S phase and declines in the G2 phase.[2,3] TK-1 phosphorylates thymidine (dThd) and 2′-deoxyuridine (dUrd).[4] TK-2 is a mitochondrial enzyme[5] and represents only a small fraction of the cellular TK levels in proliferating cells. However, TK-2 has been suggested to be responsible for most of the TK activity in resting or terminally differentiated cells (i.e., monocytes/macrophages). TK-2 not only recognizes dThd and dUrd but also efficiently phosphorylates 2′-deoxycytidine (dCyd). It should be pointed out that TK-2 resembles more the herpes simplex virus thymidine kinase (HSV-TK) than human TK-1, both in substrate specificity and in primary sequence.[6]

As enzymes involved in the activation of pyrimidine nucleosides and nucleoside analogues, thymidine kinases are an important area of research in both the anticancer and antiviral fields. The role of TK-2 is particularly under debate. First, its low level of expression, its mitochondrial localization, and the concomitant presence of the cytosolic TK-1 make it difficult to evaluate its contribution either in mitochondrial or in cellular events, as well as in activation of antiviral or anticancer nucleoside analogues. Recently, mutant TK-2 was found to be the genetic basis for the mitochondrial DNA depletion in the hereditary disorder of several individuals who suffered from severe devastating myopathy.[7] This points indeed to a crucial role of TK-2 in mitochondrial DNA synthesis. Second, it has been proposed that the mitochondrial toxicity observed under prolonged treatments with the anti-hepatitis B virus nucleoside analogue FIAU[8] or with the anti-HIV drug AZT[9] is due to activation of these nucleoside analogues by TK-2. Therefore, the use of TK-2 inhibitors may help to resolve fundamental questions such as the physiological role of TK-2 and may also reveal and clarify the real contribution of TK-2-catalyzed phosphorylation of antiviral drugs in their mitochondrial toxicity. Therefore, there is an increasing interest and need for specific human mitochondrial thymidine kinase inhibitors.[10,11]

Our approach to the identification of new TK-2 inhibitors has relied on the evaluation of nucleoside analogues previously synthesized in our laboratories[12] or easily obtained from commercial sources. From this last approach, it was found that 5′-*O*-(4,4′-dimethoxytrityl)thymidine (DMTr-dThd, **1**)[13] showed slight inhibitory activity against TK-2-catalyzed dThd phosphorylation ($IC_{50} = 468 \pm 45$ μM). This compound was 1 order of magnitude more active against the related

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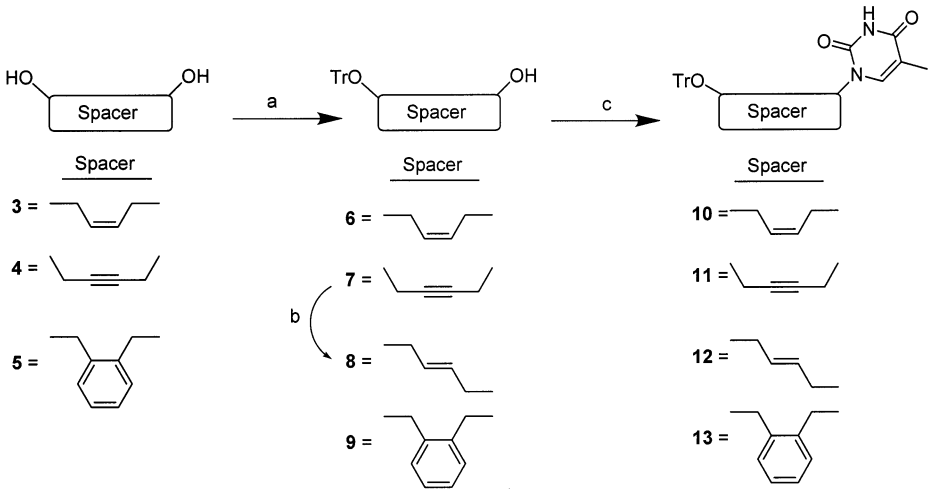
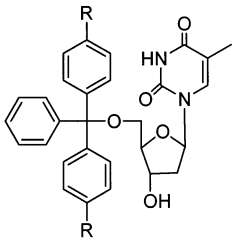
Scheme 1[a]



[a] (a) TrCl, DMAP, Et_3N, CH_2Cl_2. (b) $LiAlH_4$, THF, 0 °C. (c) 1, N^3-BzT, PS-Ph_3P, DIAD, THF; 2, dioxane/1 M NaOH (1:1).

Chart 1



1 R = OCH_3 5′-O-DMTr-dThd
2 R = H 5′-O-Tr-dThd

HSV-1 TK enzyme ($IC_{50} = 13.6 \pm 1.0\ \mu M$). Replacement of the strongly acid labile 4,4′-dimethoxytrityl group by the more stable trityl analogue (5′-*O*-tritylthymidine, Tr-dThd, **2**) markedly improved the inhibitory potency against both enzymes, in particular against TK-2. The presence of the trityl or dimethoxytrityl substituent at position 5′ of thymidine converts the thymidine substrate into an inhibitor because both compounds (**1** and **2**, Chart 1) lack a 5′-OH group that allows phosphorylation. This observation led us to hypothesize that the role of the sugar moiety in compounds **1** and **2** may serve only to position the base and the trityl substituents in the right way to interact with the enzyme and, thus, the sugar moiety has been replaced by acyclic spacers that may fulfill the same spatial function. Moreover, these acyclic nucleoside structures may reduce the possibility of other toxicities from accidental interference with other nucleoside-processing enzymes (i.e., TK-1).

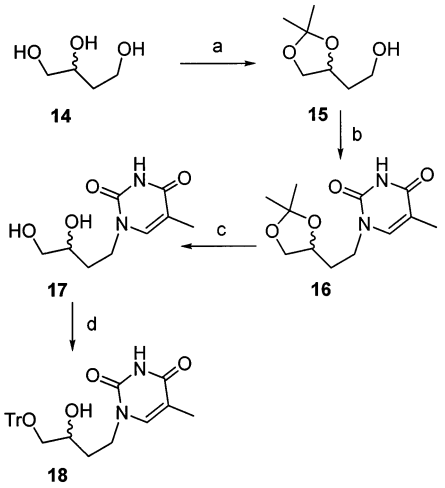
In the present study, we describe the synthesis and enzymatic evaluation of a series of acyclic nucleoside analogues of the general formula [Thy]-spacer-[trityl] against TK-2 and the phylogenetically closely related HSV-1 TK. The lead compound has been further explored and optimized by replacing or modifying the thymine base and the trityl substituent. Due to the difficulties encountered in evaluating TK-2 inhibitors in cell culture,[14] and based on the similarities in substrate specificity between TK-2 and HSV-1 TK, the most potent inhibitors in the enzymatic assays (i.e., **10** and **12**) have been studied in a human osteosarcoma cell line (OST-TK^-/HSV-1 TK^+) that is transduced by and expresses the HSV-1 TK gene.

Chemistry

One of the very well-known strategies in nucleoside analogue synthesis is the replacement of the sugar moiety by conformationally constrained acyclic chains.[15] Among them, residues containing butynols or (*E*)- or (*Z*)-butenols are widely used.[16,17] Alkylation of the thymine moiety with acyclic chains through their corresponding tosylates, mesylates, or bromides is hampered, in general, by low yields of the coupling products. The Mitsunobu condensation employing N^3-benzoylthymine (N^3-BzT) represents an interesting alternative.[18] However, it also suffers from some drawbacks mostly due to the difficult elimination of the excess of reagents used (Ph_3P, DIAD) and the byproducts of the reaction, in particular Ph_3PO, that make difficult not only the isolation of the final compounds but also the follow-up of the reaction. The use of polystyrene-bound triphenylphosphine (PS-triphenylphosphine), prepared by Ford[19] and now commercially available, can be very helpful to simplify isolation of the target compounds, because both the excess of Ph_3P and the generated Ph_3PO remained attached to the resin and can be removed by simple filtration.

Therefore, our approach to the synthesis of the [Thy]-spacer-[trityl] series, shown in Scheme 1, has been the introduction of the thymine base on tritylated spacers under Mitsunobu conditions. The intermediate alcohols **6**−**9** were obtained by monotritylation of commercially available diols **3**−**5**, with the exception of **8**, the corresponding diol of which is not commercially available. This (*E*)-buten-2-yl derivative **8**[20] was prepared by reduction of the butynyl alcohol **7** with $LiAlH_4$. Condensation of the alcohols **6**−**9** with N^3-BzT[21] in the presence of PS-triphenylphosphine and DIAD in dry THF, followed by debenzoylation by treatment with 1 M NaOH in a dioxane/H_2O (1:1) solution, afforded the target compounds **10**−**13** in good yields (61−89%). Under these reaction conditions, the excess of thymine is retained in the aqueous phase and the target compounds **10**−**13** remain in the organic phase and are easily isolated by chromatography. In every case, the only condensation product detected was the alkyl compound at position 1 of the thymine ring.

Scheme 2[a]



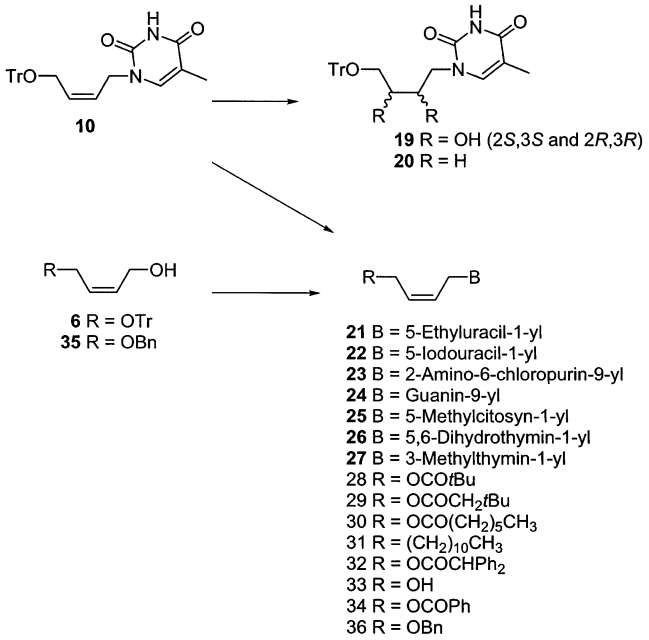
[a] (a) Acetone, pTSA, rt. (b) 1, N^3-BzT, PS-Ph_3P, DIAD, THF; 2, dioxane/1 M NaOH (1:1). (c) AcOH 80%, Δ. (d) TrCl, DMAP, Py, Δ.

To increase the conformational flexibility on the spacer and/or to include hydroxyl groups that could mimic the OH of the (2-deoxy)ribose moiety of the parent nucleosides (**1** and **2**), a second series of modifications were carried out. The synthesis of the 2-deoxyribose mimetic (**18**) is shown in Scheme 2. On the basis of the good yields obtained under Mitsunobu conditions, this was also the key step in this reaction sequence. Thus, treatment of 1,2,4-butanetriol (**14**) with *p*-toluenesulfonic acid in acetone, under described conditions, afforded the five-membered ring acetonide **15**.[22] Reaction of this alcohol (**15**) with N^3-BzT[21] under Mitsonubu conditions (PS-triphenylphosphine, DIAD) followed by debenzoylation gave the N-1 alkyl compound **16**. Removal of the isopropylidene moiety by treatment with 80% AcOH afforded the diol **17** (40% yield starting from **14**). Previous synthesis of this compound by Holy,[23] following a two-step procedure, gave **17** in 23% yield. Tritylation of the primary alcohol in **17** afforded the target compound **18**.

The (*Z*)-butenyl derivative **10** was used to obtain the saturated and bis-hydroxylated derivatives in the spacer (Scheme 3). Thus, treatment of **10** with OsO_4 in the presence of *N*-methylmorpholine-*N*-oxide afforded the enantiomeric mixture of *cis*-diols **19** (67% yield). On the other hand, reduction of **10** by catalytic hydrogenation (H_2, 10% Pd/C) yielded the butyl derivative **20** in 47% yield. When compounds **10**−**13** and **18**−**20** were evaluated against TK-2 and HSV-1 TK (see Biological Results and Discussion), it was found that the (*Z*)-butenyl derivative **10** showed the most potent inhibition against TK-2, being 1 order of magnitude more potent than the parent nucleoside (**2**). Therefore, a new series of modifications were performed now using compound **10** as the lead, keeping the (*Z*)-butenyl as the spacer and replacing or modifying the thymine base and the trityl susbtituent.

Reaction of the synthon **6** (Scheme 3) with N^3-benzoyl-5-ethyluracil[21,24] or N^3-benzoyl-5-iodouracil[21,24] in the presence of PS-triphenylphosphine and DIAD, followed by treatment with 1M NaOH in dioxane/water (1:1) gave the N-1 alkyl derivatives of 5-ethyl and 5-iodouracil, **21** and **22**, in 50 and 30% yields, respectively. On the other hand, reaction of the alcohol **6** with 6-amino-2-chloro-

Scheme 3



purine, under the same reaction conditions, yielded the coupling product at position 9 of the purine ring (**23**, 87% yield). Treatment of **23** with 1 N NaOH in dioxane[25] afforded the guanine derivative **24** in 84% yield.

Derivative **10** was used to perform some modifications on the thymine ring (Scheme 3). Thus, reaction of **10** with $POCl_3$ and 1,2,4-triazole in Et_3N and acetonitrile,[26] followed by treatment with NH_4OH in dioxane, gave the 5-methylcytosine derivative **25** (21% yield from **10**). On the other hand, the 5,6-dihydrothymine derivative **26** was obtained in 54% yield by a novel procedure that involved reduction of the pyrimidine ring with DIBAL-H in THF, as an interesting alternative to described procedures that perform such reduction by catalytic hydrogenation in the presence of the expensive Rh catalysts.[27] By avoiding the catalytic hydrogenation, the double bond of the spacer remains unaltered and the trityl substituent is not removed. Treatment of **10** with CH_3I in the presence of K_2CO_3 leads to the N^3-methylthymine derivative **27** in 93% yield.

Finally, replacement of the trityl group in the lead compound **10** by other lipophilic moieties, both aromatic and aliphatic, was assayed. Because the inhibitory capacity of the synthesized compounds would be tested in a cell-free assay using purified enzyme, it was considered that ester derivatives, which are easily synthesized, would provide interesting information about the structural requirements for inhibitory activity. In a very recent paper, a one-step method for the deprotection and esterification of trityl ethers has been described by treatment of the trityl ether with a large excess (200−1000 equiv) of the corresponding acyl chloride.[28] When these reaction conditions were assayed on the trityl ether **10** using pivaloyl chloride (100 equiv) as the acylating agent, only extensive decomposition was observed. In the above-mentioned paper, it is proposed that the large excess of the acyl chloride could be required to ensure the presence of enough HCl in the reaction medium to perform the deprotection of the trityl ethers.[28] As an alternative to this acid medium, addition of an acid resin (Dowex 50WX4, H^+ form) was

Table 1. Inhibitory Effect of the Synthesized Compounds on the Phosphorylation of [*methyl*-^{3}H]dThd by TK-2 and HSV-1 TK

	IC_{50}[a,b] (μM)			IC_{50}[a,b] (μM)	
compd	TK-2	HSV-1 TK	compd	TK-2	HSV-1 TK
1	NEA[c]	14 ± 1.0	**25**	NEA[c]	NEA[c]
2	33 ± 20	7.8 ± 0.3	**26**	9.8 ± 1.9	NEA[c]
10	1.5 ± 0.16	45 ± 1	**27**	NEA[c]	NEA[c]
11	NEA[c]	3.1 ± 0.2	**28**	40 ± 11	NEA[c]
12	25 ± 13	3.0 ± 0.0	**29**	28 ± 5	NEA[c]
13	NEA[c]	NEA[c]	**30**	11 ± 7	NEA[c]
18	3.6 ± 0.4	1.2 ± 0.7	**31**	19 ± 12	NEA[c]
19	17 ± 0.3	13 ± 1.5	**32**	4.6 ± 0.5	NEA[c]
20	3.3 ± 1.2	10 ± 1	**33**	NEA[c]	NEA[c]
21	20 ± 7	NEA[c]	**34**	30 ± 4	NEA[c]
22	4.6 ± 0.4	48 ± 14	**36**	22 ± 4	NEA[c]
24	NEA[c]	NEA[c]			

[a] 50% inhibitory concentration or compound concentration (expresed in μM) required to inhibit dThd phosphorylation by 50%. Data are the mean value (± SD) of at least two to three independent experiments [b] IC_{50} values were obtained in the presence of 10% DMSO at each inhibitor concentration, including the control assays (in the absence of inhibitor). [c] NEA, not estimated accurately. No inhibition of enzyme activity was noted at any drug concentration used (up to 500 μM). However, because full solubility of the drug could not be assured at 500 and 50 μM (even in the presence of 1% DMSO), accurate values at which inactivity has been shown cannot be given.

assayed, and under these conditions the number of equivalents of acyl chloride required was reduced. Thus, treatment of **10** (Scheme 3) with pivaloyl chloride (50 equiv) in CH_2Cl_2 and in the presence of Dowex 50WX4 (H^+ form) (100 equiv) led to the acyl derivative **28** in 66% yield. This procedure allows the easy isolation of the reaction product **28** by filtration of the resin, hydrolysis of the excess of acyl chloride, washing with aqueous $NaHCO_3$, and a short chromatography. Following this procedure, compound **10** was easily transformed into the acyl derivatives **29**−**32** carrying *tert*-butylacetyl, hexanoyl, decanoyl, and diphenylacetyl moieties, respectively (Scheme 3). It is interesting to mention that reaction of **10** with benzoyl chloride, under the same reaction conditions, gave only traces of the benzoyl derivative (**34**). This is in agreement with the published results[28] which indicate that aromatic acyl chlorides afford only low yields of the corresponding acyl products. Alternatively, the *O*-benzoyl compound[29] was prepared by treatment of **10** with Dowex 50WX4 (H^+ form) in MeOH to yield the free OH[17] (**33**) (98% yield), which by treatment with benzoyl chloride in pyridine afforded the *O*-benzoyl derivative **34** in 53% yield.

Finally, the *O*-benzyl derivative **36** was prepared in 59% yield by condensation of the benzyl alcohol **35**[30] with N^3-BzT under Mitsunobu conditions.

Biological Results and Discussion

Inhibition of [*methyl*-^{3}H]dThd Phosphorylation by TK-2 and HSV-1 TK. Compounds **10**−**13**, **18**−**22**, and **24**−**36** have been tested for their inhibitory effect against phosphorylation of 2 μM dThd by recombinant TK-2 and HSV-1 TK (see Experimental Section), and the results are shown in Table 1. Compounds **1** and **2** are also included in the table for comparative reasons. Evaluation of the first series of compounds (**10**−**13** and **18**−**20**) indicated that replacement of the furanosyl moiety in compounds **1** and **2** by aliphatic chain spacers, according to our working hypothesis, not only kept the inhibitory capacity of several derivatives but further increased the inhibitory potency against the target enzymes. Thus, derivative **12** containing an (*E*)-butenyl spacer showed a behavior similar to that of compound **2**, being slightly more active than **2** against HSV-1 TK. Replacement of the furanosyl moiety by a (*Z*)-butenyl spacer (**10**) increased the inhibitory potency 20-fold against TK-2, but at the same time reduced the inhibitory potency 6-fold against HSV-1 TK. The butynyl derivative **11** showed a very weak activity at the level of TK-2 while being as potent as compound **12** against HSV-1 TK. Thus, in this case, there is a marked specificity of compound **11** for HSV-1 TK. The benzylic derivative **13** was completely inactive against both enzymes. By increasing the conformational freedom in the spacer, as shown for the butyl derivatives **18**−**20**, potent inhibitors were obtained against both enzymes and the enzyme selectivity was reduced. From the above-mentioned data, it was concluded that the (*Z*)-butenyl derivative **10** was the most potent and selective compound against TK-2 in this series.

Further modifications performed at the base level revealed that this fragment is crucial for the interaction of this family of nucleoside kinase inhibitors. Replacement of the thymine base in compound **10** by guanine (**24**) or by 5-methylcytosine (**25**) abolished the inhibitory capacity. Methylation of position 3 of the thymine ring, as shown in compound **27**, resulted in complete inactivity. Substitution of the thymine base by the close analogue 5-iodouracil (**22**) kept activity against both enzymes, and the inhibitory behavior was quite similar to that of the lead compound **10**. The 5-ethyluracil derivative (**21**) was 1 order of magnitude less potent against TK-2 and lost inhibitory activity against HSV-1 TK. It should also be pointed out that the 5,6-dihydrothymine derivative (**26**) exclusively showed activity against TK-2 with an IC_{50} value of 9.8 μM, therefore being a highly selective TK-2 inhibitor.

In the last series of modifications, the trityl moiety was replaced by aromatic and aliphatic acyl residues. All compounds in the acyl series were inactive or almost inactive against the herpes TK enzyme while moderately active against TK-2. Among them, the diphenyl acetyl derivative **32** showed the most potent inhibition of TK-2 (IC_{50} = 4.6 μM). It should also be pointed out that compound **33** shows an IC_{50} of ∼200 μM against TK-2, stressing the importance of the presence of a highly lipophilic entity, preferentially a diphenylmethyl or triphenylmethyl moiety, for enzyme inhibition. Finally, the *O*-benzoyl and *O*-benzyl derivatives (**34** and **36**) were equally inhibitory to TK-2 (IC_{50} = 22−30 μM), being inactive against HSV-1 TK.

The most potent TK-2 inhibitors, including compounds **10**, **12**, **18**, and **26**, were also tested for their inhibitory activity against cytosolic TK-1-catalyzed phosphorylation of the natural substrate dThd. All of them showed no inhibitory activity against TK-1 at 100 μM.

Inhibition of HSV-1 TK-Catalyzed [^{3}H]GCV Phosphorylation by Compound 10. From the above-discussed data, it became clear that the base part of this new family of nucleoside kinase inhibitors played a crucial role in the interaction with their target enzymes. Because no crystallographic X-ray structure has been available for TK2, it is difficult to speculate how these

Table 2. Inhibition of HSV-1 TK-Catalyzed [^{3}H]GCV Phosphorylation by dThd, dGuo, and Compounds **10** and **12**

	50% inhibitory concentration[a] (μM)	
compd	HSV-1 TK (WT)	HSV-1 TK (A167Y)
dThd	1.8 ± 1.0	≥500
dGuo	364 ± 90	115 ± 11
10	8.7 ± 2.5	>50
12	0.39 ± 0.07	48 ± 3.2

[a] 50% inhibitory concentration or compound concentration, expressed in μM, required to inhibit [^{3}H]GCV phosphorylation by 50%.

inhibitors fit into the enzyme. However, the crystal structures of HSV-1 TK complexed with either the natural substrate dThd or antiherpetic drugs such as the guanine nucleoside analogue ganciclovir (GCV) have been resolved and revealed that both pyrimidine and purine nucleoside analogues bind to the same substrate binding site of the enzyme. On the basis of these crystallographic structures, we have recently designed a mutant HSV-1 TK (A167Y) that discriminates between purine and pyrimidine nucleoside substrates.[31] Indeed, as shown in Table 2, dThd and, to a lesser extent, dGuo inhibit [^{3}H]GCV phosphorylation by the wild-type HSV-1 TK, presumably by competitive inhibition. However, pyrimidine nucleosides, such as dThd, are not recognized by the mutant enzyme (A167Y) either as substrates or as inhibitors (the mutant enzyme does not have a decreased GCV phosphorylation), thus not affecting GCV phosphorylation by the mutant enzyme. Therefore, recognition of dThd by the mutant (A167Y) HSV-1 TK enzyme is seriously compromised. In contrast, GCV phosphorylation by the mutant enzyme is equally affected by dGuo as the wild-type enzyme. Interestingly, when compound **10**, a thymine-derived TK-2/HSV-1 TK inhibitor, was investigated against the wild-type and the mutant HSV-1 TK enzymes, its inhibitory capacity against GCV phosphorylation by the wild-type enzyme was very pronounced, whereas GCV phosphorylation by the mutant enzyme was unaffected by the compound. This could indicate that the thymine-based TK inhibitors such as **10** interact with HSV-1 TK in the substrate binding site and may bind to the enzyme in a fashion similar to that of the natural substrate dThd.

Evaluation of 10, 12, and 13 in the OST-TK$^-$/HSV-1 TK$^+$ Cell Lines. Direct evaluation of TK-2 inhibitors in cell culture has proven to be difficult, and alternative ways to express TK-2 in tumor cells and to evaluate the inhibitors in an intact cell system have been proposed.[14] However, a tumor cell line expressing TK-2 in the cytosol is not available yet. On the basis of the existing similarities between TK-2 and HSV-1 TK, and because some of our most potent enzyme inhibitors showed inhibitory activity against both enzymes, we have investigated two of these inhibitors (**10** and **12**) and one derivative that show no inhibitory activity against the enzyme (**13**) in OST-TK$^-$/HSV-1 TK$^+$ cell lines in combination with HSV-1 TK substrates, such as ganciclovir (GCV) and (*E*)-5-(2-bromovinyl)-1-β-D-(arabinofuranosyl)uracil (BVaraU) (see Table 3). The OST-TK$^-$/HSV-1 TK$^+$ cells represent human osteosarcoma cells that are deficient for cytosolic TK but express HSV-1 TK in the cytosol.[32] When the substrates GCV and BVaraU were exposed to OST-TK$^-$/HSV-1 TK$^+$ cell cultures, a marked cytotoxicity was observed with IC_{50} values in the low nanomolar range (0.004−0.008 μM). Addition of HSV-1 TK inhibitors, such as compound **10** or **12** at a concentration of 20 μM (see Experimental Section), to these cell cultures allowed a marked reduction of 3 orders of magnitude in the cytotoxic effect of both GCV and BVaraU. This "detoxifying" effect is more pronounced and efficient by the addition of these inhibitors than by the addition of thymidine as the natural substrate. In contrast, compound **13** that showed no inhibitory effect against HSV-1 TK did not change the inhibitory values of GCV and BvaraU (Table 3). Thus, potential alterations of the cell membrane properties by the lipophilic compounds affecting uptake and/or efflux of the drugs cannot be the reason for the reversing activity of **10** and **12**. Although compound **12** is a more potent inhibitor of HSV-1 TK than compound **10**, it reversed the cytostatic activity of GCV and BVaraU to a similar extent as compound **10**. Although puzzling, it is not known whether the administered drug concentrations (20 μM for **10** and **12**) are at saturating concentrations to allow full inhibition of the enzyme in the cytoplasma because no dose−response experiment has been performed. However, the main purpose of this experiment was to prove whether compounds **10** and **12** may enter the cells and inhibit cytosolic HSV-1 TK at subtoxic concentrations, and this turned out to be the case. These results support the potential interest of the synthesized inhibitors such as **10** or **12** to be combined in cell culture with TK-dependent nucleoside analogues to avoid or prevent potential toxic side effects.

Conclusions

A series of acyclic nucleoside analogues of 5′-*O*-tritylthymidine have been synthesized and tested against human mitochondrial thymidine kinase (TK-2) and herpes simplex virus TK (HSV-1 TK).

Replacement of the sugar moiety by acyclic chains resulted in inhibitors that were 1 order of magnitude more potent than the parent 5′-*O*-tritylthymidine. Among them, the (*Z*)-butenyl derivative (**10**) showed an IC_{50} against TK-2 of 1.5 μM.

To the best of our knowledge, the here-described inhibitors represent the first example of nucleoside analogues lacking a (deoxy)ribose moiety that show pronounced inhibition of human mitochondrial thymidine kinase 2. Finally, the results obtained in the cell culture model (OST-TK$^-$/HSV-1 TK$^+$ cells) combining

Table 3. Effect of dThd and Inhibitors of HSV-1 TK on the Cytostatic Activity of GCV and BVAraU in OST-TK$^-$/HSV-1 TK$^+$ Cell Lines

	IC_{50}[a] (μM)				
	as such	+ **10** (20 μM)	+ **12** (20 μM)	+ **13** (20 μM)	+ dThd (50 μM)
GCV	0.004 ± 0.002	0.18 ± 0.17	0.23 ± 0.19	0.002 ± 0.0003	0.026± 0.002
BVAraU	0.008 ± 0.006	2.2 ± 1.6	3.1 ± 0.85	0.008 ± 0.001	0.016± 0.009

[a] 50% inhibitory concentration or compound concentration, expressed in μM, required to inhibit cell proliferation by 50%.

HSV-1 TK substrates and the here-reported inhibitors demonstrate that TK inhibitors are efficiently taken up by intact cells to reach the cytosolic compartment and are sufficiently stable and potent to exert their inhibitory activity in intact cells. These data also support the potential of this new family of compounds to avoid or prevent the toxic effects observed with TK-dependent nucleoside analogues.

Experimental Section

Chemical Procedures. Melting points were obtained on a Reichert-Jung Kofler apparatus and are uncorrected. Microanalyses were obtained with a Heraeus CHN-O-RAPID instrument. Electrospray mass spectra were measured on a quadrupole mass spectrometer equipped with an electrospray source (Hewlett-Packard, LC/MS HP 1100). ^{1}H and ^{13}C NMR spectra were recorded on a Varian Gemini operating at 200 MHz (^{1}H) and 50 MHz (^{13}C), respectively, on a Varian INNOVA 300 operating at 299 MHz (^{1}H) and 75 MHz (^{13}C), respectively, and on a Varian INNOVA 400 operating at 399 MHz (^{1}H) and 99 MHz (^{13}C), respectively. Monodimensional ^{1}H and ^{13}C spectra were obtained using standard conditions. Two-dimensional inverse proton detected heteronuclear one-bond shift correlation spectra were obtained using the pulsed field gradient HSQC pulse sequence.

Analytical TLC was performed on silica gel 60 F_{254} (Merck) precoated plates (0.2 mm). Spots were detected under UV light (254 nm) and/or by charring with phosphomolybdic acid. Separations on silica gel were performed by preparative centrifugal circular thin-layer chromatography (CCTLC) on a Chromatotron [Kiesegel 60 PF_{254} gipshaltig (Merck)], layer thickness = 1 or 2 mm, flow rate = 4 or 8 mL/min, respectively. Flash column chromatography was performed with silica gel 60 (230−400 mesh) (Merck).

Polystyrene−triphenylphosphine (PS-Ph_3P) was purchased from Fluka. Diisopropyl azodicarboxylate (DIAD) was purchased from Aldrich.

Triethylamine and acetonitrile were dried by refluxing over calcium hydride. Tetrahydrofuran was dried by refluxing over sodium/benzophenone. Anhydrous *N*,*N*′-dimethylformamide was purchased from Aldrich.

The radiolabeled substrates [*CH*$_3$-^{3}H]dThd (70 Ci/mmol) and [2,8-^{3}H]ganciclovir (12.4 Ci/mmol) were obtained from Moravek Biochemicals (Brea, CA).

General Procedure for Monotritylation of Diols. Synthesis of 6, 7, and 9. To a stirred solution of the corresponding diol (**3**−**5**) (10 mmol) in dry CH_2Cl_2 (4 mL) at 0 °C were added Et_3N (0.15 mL, 1.1 mmol), DMAP (5 mg, 0.04 mmol), and trityl chloride (278 mg, 1.0 mmol). The mixture was stirred at room temperature for 24 h. Then it was diluted with CH_2Cl_2 (20 mL) and water (10 mL). The organic phase was washed with water (10 mL) and brine (10 mL). The organic layer was dried on anhydrous Na_2SO_4, filtered, and evaporated. The residue was purified by flash column chromatography or in the Chromatotron as indicated.

(*Z*)-1-Hydroxy-4-(triphenylmethoxy)-2-butene (6) was p repared from (*Z*)-2-butene-1,4-diol (**3**) and purified by CCTLC in the Chromatotron (hexane/EtOAc, 1:1): yield, 80%; ^{1}H NMR was identical to that described.[33]

1-Hydroxy-4-(triphenylmethoxy)-2-butyne (7) was prepared from 2-butyne-1,4-diol (**4**) and purified by CCTLC in the Chromatotron (hexane/EtOAc, 3:1): yield, 50% of a white solid; mp 74−76 °C; MS (ES, positive mode), *m*/*z* 451 (M + Na)$^+$; ^{1}H NMR ($CDCl_3$) δ 1.56 (t, J = 5.7 Hz, 1H, OH), 3.83 (s, 2H, CH_2-OTr), 4.26 (d, J = 5.7 Hz, 2H, *CH*$_2$OH), 7.25−7.49 (m, 19H, Ph); ^{13}C NMR ($CDCl_3$) δ 51.20, 53.05 (CH_2OH, CH_2OTr), 82.50, 83.57 (C≡C), 87.46 (*C*Ph$_3$), 127.15, 127.92, 128.59, 134.38 (Ph).

(*E*)-1-Hydroxy-4-(triphenylmethoxy)-2-butene (8). A suspension of $LiAlH_4$ (48 mg, 1.2 mmol) in dry THF (1.2 mL) was cooled to 0 °C in an ice bath. A solution of **7** (150 mg, 0.46 mmol) in dry THF (7.6 mL) was added, and the resulting suspension was stirred at 4 °C for 2 h and at room temperature for an additional hour. After cooling to 0 °C, Na_2SO_4 (85 mg, 0.60 mmol) and water (0.11 mL, 6 mmol) were added and stirring was continued overnight. Then CH_2Cl_2 (40 mL) and brine (20 mL) were added. The organic phase was dried on anhydrous Na_2SO_4, filtered, and evaporated. The residue was purified by CCTLC in the Chromatotron (hexane/EtOAc, 1:1) to yield 146 mg (96%) of **8** as a solid: mp 62−63 °C (lit.[20] 61−62 °C); ^{1}H NMR was identical to that described.[20]

(2-Triphenylmethoxyphenyl)methanol (9) was prepared from 1,2-bis(hydroxymethyl)benzene (**5**) according to the general procedure for monotritylation of diols. The residue was purified by column chromatography (hexane/EtOAc, 1:1): yield, 83%; mp 102−105 °C; MS (ES, positive mode), *m*/*z* 403 (M + Na)$^+$; ^{1}H NMR ($CDCl_3$) δ 2.89 (t, J = 6.6 Hz, 1H, OH), 4.13 (s, 2H, CH_2OTr), 4.37 (d, J = 6.4 Hz, 2H, *CH*$_2$OH), 7.25−7.41 (m, 19H, Ph).

General Procedure for the Mitsunobu Condensation of Alcohols with *N*^{3}-Benzoylthymine. To a suspension containing the corresponding alcohol (**6**−**9**) (0.5 mmol), PS-Ph_3P (3 mmol/g, 416 mg, 1.25 mmol), and N^3-BzT (230 mg, 1.0 mmol) in dry THF (5 mL) was slowly added a solution of DIAD (0.19 mL, 1.0 mmol) in dry THF (2 mL). The mixture was stirred at room temperature overnight. The reaction was filtered, the residue was washed with THF (2 × 5 mL), and the combined filtrates were evaporated to dryness. The residue was dissolved in a dioxane/NaOH 1 M (1:1) mixture (9 mL) and stirred overnight. Then, EtOAc (10 mL) and brine (5 mL) were added. The aqueous phase was further extracted with EtOAc (3 × 10 mL). The combined organic extracts were dried on anhydrous Na_2SO_4, filtered, and evaporated. The residue was purified by CCTLC in the Chromatotron (hexane/EtOAc, 1:1) to yield the target compounds.

1-[(*Z*)-4-(Triphenylmethoxy)-2-butenyl]thymine (10) was prepared from alcohol **6** (165 mg, 0.50 mmol) according to the general procedure: yield, 82%; mp 148−150 °C; MS (ES, positive mode), *m*/*z* 461 (M + Na)$^+$; ^{1}H NMR ($CDCl_3$) δ 1.91 (s, 3H, 5-CH_3), 3.72 (d, J = 6.9 Hz, 2H, CH_2O), 4.18 (d, J = 7.1 Hz, 2H, CH_2N), 5.56, 5.95 (m, 2H, CH=CH), 6.86 (s, 1H, H-6), 7.23−7.46 (m, 15H, Ph), 7.94 (br s, 1H, 3-NH); ^{13}C NMR ($CDCl_3$) δ 12.23 (5-CH_3), 44.28 (CH_2N), 59.55 (CH_2O), 87.29 (*C*Ph$_3$), 110.86 (C-5), 125.86, 131.69 (CH=CH), 127.21, 127.96, 128.53, 143.69 (Ph), 139.53 (C-6), 150.53 (C-2), 164.00 (C-4). Anal. for $C_{28}H_{26}N_2O_3$: C, H, N.

1-[4-(Triphenylmethoxy)-2-butynyl]thymine (11) was prepared from alcohol **7** (179 mg, 0.50 mmol) according to the general procedure: yield, 61%; mp 80−82 °C; MS (ES, positive mode), *m*/*z* 459 (M + Na)$^+$; ^{1}H NMR ($CDCl_3$) δ 1.92 (s, 3H, 5-CH_3), 3.83 (t, J = 1.8 Hz, 2H, CH_2O), 4.49 (t, J = 1.8 Hz, 2H, CH_2N), 7.17 (s, 1H, H-6), 7.23−7.34 (m, 15H, Ph), 8.43 (br s, 1H, 3-NH); ^{13}C NMR ($CDCl_3$) δ 12.43 (5-CH_3), 36.97 (CH_2N), 52.98 (CH_2O), 77.68, 83.80 (HC≡CH), 87.70 (*C*Ph$_3$), 111.18 (C-5), 127.29, 127.97, 128.53, 143.21 (Ph), 138.55 (C-6), 150.22 (C-2), 163.75 (C-4). Anal. for $C_{28}H_{24}N_2O_3$: C, H, N.

1-[(*E*)-4-(Triphenylmethoxy)-2-butenyl]thymine (12) was prepared from alcohol **8** (100 mg, 0.30 mmol) according to the general procedure: yield, 85%; amorphous solid; MS (ES, positive mode), *m*/*z* 461 (M + Na)$^+$; ^{1}H NMR ($CDCl_3$) δ 1.90 (s, 3H, 5-CH_3), 3.64 (t, J = 2.8 Hz, 2H, CH_2O), 4.31 (t, J = 5.3 Hz, 2H, CH_2N), 5.73−5.82 (m, 2H, CH=CH), 6.93 (s, 1H, H-6), 7.21−7.40 (m, 15H, Ph), 8.19 (br s, 1H, 3-NH); ^{13}C NMR ($CDCl_3$) δ 12.32 (5-CH_3), 48.97 (CH_2N), 63.71 (CH_2O), 87.14 (*C*Ph$_3$), 110.86 (C-5), 124.09, 132.54 (CH=CH), 127.13, 127.88, 128.59, 143.93 (Ph), 139.65 (C-6), 150.51 (C-2), 163.71 (C-4). Anal. for $C_{28}H_{26}N_2O_3$: C, H, N.

1-[2-((Triphenylmethoxy)methyl)benzyl]thymine (13) was prepared from alcohol **9** (190 mg, 0.50 mmol) according to the general procedure: yield, 67%; mp 103−105 °C; MS (ES, positive mode), *m*/*z* 511 (M + Na)$^+$; ^{1}H NMR ($CDCl_3$) δ 1.73 (s, 3H, 5-CH_3), 4.14 (s, 2H, CH_2O), 4.71 (s, 2H, CH_2N), 6.76 (s, 1H, H-6), 7.13−7.50 (m, 19H, Ph), 8.67 (br s, 1H, 3-NH); ^{13}C NMR ($CDCl_3$) δ 12.25 (5-CH_3), 47.16 (CH_2N), 63.82 (CH_2O), 87.60 (*C*Ph$_3$), 110.89 (C-5), 127.27, 128.00, 128.42, 128.54, 129.46, 133.96, 136.58, 143.57 (Ph), 139.69 (C-6), 151.09 (C-2), 163.97 (C-4). Anal. for $C_{32}H_{28}N_2O_3$: C, H, N.

(*RS*)-1-[2-(2,2-Dimethyl-[1,3]dioxolan-4-yl)ethyl]thymine (16) was prepared from alcohol **15**[22] (100 mg, 0.68 mmol) according to the general procedure: yield, 67%; mp 108−110 °C; MS (ES, positive mode), *m*/*z* 255 $(M + 1)^+$, 277 $(M + Na)^+$; ^{1}H NMR ($CDCl_3$) δ 1.31, 1.38 [2s, 6H, $(CH_3)_2C$], 1.89 (s, 3H, 5-CH_3), 1.81, 1.99 (m, 2H, CH_2), 3.53, 4.07 (m, 2H, CH_2O), 3.71, 3.93 (m, 2H, CH_2N), 4.05 (m, 1H, CH), 7.05 (s, 1H, H-6), 9.53 (br s, 1H, 3-NH); ^{13}C NMR ($CDCl_3$) δ 12.23 (5-CH_3), 25.47, 26.92 [$(CH_3)_2C$], 32.42 (CH_2), 45.92 (CH_2N), 69.03 (CH_2O), 72.65 (CH), 109.23 [$(CH_3)_2C$], 110.42 (C-5), 140.94 (C-6), 150.98 (C-2), 164.49 (C-4). Anal. for $C_{12}H_{18}N_2O_4$: C, H, N.

(*RS*)-1-[3-Hydroxy-4-(triphenylmethoxy)butyl]thymine (18). A solution of **16** (100 mg, 0.40 mmol) in 80% acetic acid (6 mL) was heated at 80 °C for 1 h. Volatiles were removed, and the residue was coevaporated with EtOH (4 × 10 mL) and pyridine (2 × 5 mL). The residue, containing (*RS*)-1-(3,4-dihydroxybutyl)thymine[23] (**17**) was dissolved in pyridine (3 mL), and DMAP (catalytic amount) and trityl chloride (181 mg, 0.64 mmol) were added. The mixture was heated at 80 °C for 24 h. Volatiles were removed, amd the residue was treated with CH_2Cl_2 (20 mL) and water (10 mL). The aqueous phase was further extracted with CH_2Cl_2 (2 × 20 mL). The combined organic extracts were dried on anhydrous Na_2SO_4, filtered, and evaporated. The residue was purified in a silica SPE cartridge eluting with CH_2Cl_2/MeOH (200:1); 73 mg (40%) of **18** were obtained: mp 183−185 °C; MS (ES, positive mode), *m*/*z* 479 $(M + Na)^+$; ^{1}H NMR ($CDCl_3$) δ 1.31 (m, 2H, CH_2), 1.89 (s, 3H, 5-CH_3), 3.12 (m, 2H, CH_2O), 3.63 (m, 1H, C*H*OH), 3.80 (m, 2H, CH_2N), 7.00 (s, 1H, H-6), 7.17−7.42 (m, 15H, Ph), 8.29 (br s, 1H, 3-NH). Anal. for $C_{28}H_{28}N_2O_4$: C, H, N.

(2*S*,3*S*)- and (2*R*,3*R*)-1-[2,3-Dihydroxy-4-(triphenylmethoxy)butyl]thymine (19). To a solution of **10** (150 mg, 0.34 mmol) in acetone/H_2O (90:10) (9 mL) were added *N*-methylmorpholine-*N*-oxide (0.071 mL of a 60% aqueous solution, 0.41 mmol) and OsO_4 (0.085 mL of a 2.5% solution in *tert*-butyl alcohol, 0.007 mmol). The mixture was stirred at room temperature for 3 days. A small portion of $Na_2S_2O_5$ was added, and then the mixture was filtered through Celite. The filtrate was evaporated and purified by CCTLC in the Chromatotron (CH_2Cl_2/MeOH, 15:1) to yield 103 mg (67%) of **19**: mp 189−191 °C; MS (ES, positive mode), *m*/*z* 495 $(M + Na)^+$; ^{1}H NMR ($CDCl_3$) δ 1.93 (s, 3H, 5-CH_3), 3.37, 3.50 (m, 2H, OH), 3.46 (m, 2H, CH_2O), 3.64, 3.82 (m, 2H, C*H*OH), 3.76, 4.05 (dd, J = 3.3, 14.3 Hz, 2H, CH_2N), 7.28 (s, 1H, H-6), 7.31−7.42 (m, 15H, Ph), 8.66 (br s, 1H, 3-NH); ^{13}C NMR ($CDCl_3$) δ 12.22 (5-CH_3), 50.72 (CH_2N), 66.90 (CH_2O), 68.91, 73.52, (CHOH), 87.84 (*C*Ph_3), 110.82 (C-5), 127.39, 128.07, 128.54, 142.41 (Ph), 143.35 (C-6), 152.58 (C-2), 163.52 (C-4). Anal. for $C_{28}H_{28}N_2O_5 \cdot 2H_2O$: C, H, N.

1-[4-(Triphenylmethoxy)butyl]thymine (20). A solution of **10** (150 mg, 0.34 mmol) in EtOH (20 mL) was hydrogenated at room temperature in the presence of 10% Pd/C (40 mg) at 30 psi for 2 h. The mixture was filtered and the filtrate purified by CCTLC in the Chromatotron (hexane/EtOAc 1:1) to give 70 mg (47%) of **20**: mp 163−166 °C; MS (ES, positive mode), *m*/*z* 463 $(M + Na)^+$; ^{1}H NMR ($CDCl_3$) δ 1.63, 1.77 (m, 4H, CH_2), 1.90 (s, 3H, 5-CH_3), 3.12 (t, J = 6.2 Hz, 2H, CH_2O), 3.66 (t, J = 6.1 Hz, 2H, CH_2N), 6.89 (s, 1H, H-6), 7.20−7.45 (m, 15H, Ph), 9.29 (br s, 1H, 3-NH); ^{13}C NMR ($CDCl_3$) δ 12.24 (5-CH_3), 25.99, 26.79 (CH_2), 48.28 (CH_2N), 62.71 (CH_2O), 86.52 (*C*Ph_3), 110.49 (C-5), 126.91, 127.73, 128.56, 140.31 (Ph), 144.12 (C-6), 150.89 (C-2), 164.31 (C-4). Anal. for $C_{28}H_{28}N_2O_3$: C, H, N.

5-Ethyl-1-[(*Z*)-4-(triphenylmethoxy)-2-butenyl]uracil (21). Following a procedure analogous to that described for the Mitsunobu condensation of alcohols with N^3-BzT, alcohol **6** (165 mg, 0.5 mmol) reacted with N^3-benzoyl-5-ethyluracil (244 mg, 1.0 mmol) to afford **21** (226 mg, 50%): mp 118−120 °C; MS (ES, positive mode), *m*/*z* 475 $(M + Na)^+$; ^{1}H NMR ($CDCl_3$) δ 1.05 (t, J = 7.5 Hz, 3H, 5-CH_2CH_3), 2.26 (q, J = 7.5 Hz, 2H, 5-CH_2CH_3), 3.74 (d, J = 6.4 Hz, 2H, CH_2O), 4.21 (d, J = 7.1 Hz, 2H, CH_2N), 5.55, 5.95 (m, 2H, CH=CH), 6.80 (s, 1H, H-6), 7.21−7.47 (m, 15H, Ph), 8.94 (br s, 1H, 3-NH); ^{13}C NMR ($CDCl_3$) δ 12.99 (5-CH_2CH_3), 20.00 (5-CH_2CH_3), 44.36 (CH_2N), 59.64 (CH_2O), 87.24 (*C*Ph_3), 116.74 (C-5), 125.83, 131.65 (CH=CH), 127.18, 127.93, 128.51, 143.66 (Ph), 138.79 (C-6), 150.72 (C-2), 163.67 (C-4). Anal. for $C_{29}H_{28}N_2O_3$: C, H, N.

5-Iodo-1-[(*Z*)-4-(triphenylmethoxy)-2-butenyl]uracil (22). Following a procedure analogous to that described for the Mitsunobu condensation of alcohols with N^3-BzT, alcohol **6** (330 mg, 1.0 mmol) reacted with N^3-benzoyl 5-iodouracil (684 mg, 2.0 mmol) to afford **22** (136 mg, 30%): mp 150−155 °C; MS (ES, positive mode), *m*/*z* 573 $(M + Na)^+$; ^{1}H NMR ($CDCl_3$) δ 3.74 (d, J = 6.4 Hz, 2H, CH_2O), 4.23 (d, J = 7.0 Hz, 2H, CH_2N), 5.50, 6.00 (m, 2H, CH=CH), 7.26−7.44 (m, 16H, Ph, H-6), 8.20 (br s, 1H, 3-NH); ^{13}C NMR ($CDCl_3$) δ 45.68 (CH_2N), 48.86 (C-5), 58.48 (CH_2O), 82.01 (*C*Ph_3), 124.03, 128.83 (CH=CH), 127.25, 127.92, 128.52, 142.28 (Ph), 146.82 (C-6), 148.51 (C-2), 159.97 (C-4). Anal. for $C_{27}H_{23}IN_2O_3$: C, H, N.

2-Amino-6-chloro-9-[(*Z*)-4-(triphenylmethoxy)-2-butenyl]purine (23). To a suspension containing alcohol **10** (150 mg, 0.34 mmol), PS-Ph_3P (3 mmol/g, 283 mg, 0.85 mmol), and 2-amino-6-chloropurine (115 mg, 0.68 mmol) in dry THF (5 mL) was slowly added a solution of DIAD (0.13 mL, 0.68 mmol) in THF (2 mL). The mixture was stirred at room temperature overnight. The reaction was filtered, the residue was washed with THF (2 × 5 mL), and the combined filtrates were evaporated to dryness. The residue was purified by CCTLC in the Chromatotron (hexane/EtOAc, 1:1) to yield 143 mg (87%) of **23**: mp 187−190 °C; MS (ES, positive mode), *m*/*z* 482 $(M)^+$; ^{1}H NMR ($CDCl_3$) δ 3.81 (d, J = 7.4 Hz, 2H, CH_2O), 4.53 (d, J = 5.9 Hz, 2H, CH_2N), 4.91 (br s, 2H, NH_2), 5.68, 5.94 (m, 2H, CH=CH), 7.26−7.43 (m, 15H, Ph), 7.62 (s, 1H, H-8); ^{13}C NMR ($CDCl_3$) δ 48.50 (CH_2N), 60.02 (CH_2O), 87.22 (*C*Ph_3), 124.46, 132.32 (CH=CH), 125.05 (C-5), 127.15, 127.90, 128.51, 143.67 (Ph), 141.83 (br s, C-8), 151.10 (C-4), 153.48 (C-2), 158.97 (C-6). Anal. for $C_{28}H_{24}ClN_5O$: C, H, N.

9-[(*Z*)-4-(Triphenylmethoxy)-2-butenyl]guanine (24). To a solution of **23** (150 mg, 0.31 mmol) in 1,4-dioxane (23 mL) was added 1 N sodium hydroxide (14.3 mL). The mixture was stirred at 95 °C for 27 h, neutralized with acetic acid, and concentrated to dryness. The residue was purified by flash column chromatography (CH_2Cl_2/MeOH, 10:1) to yield 117 mg (81%) of **24** as a white solid: mp 252−255 °C; MS (ES, positive mode), *m*/*z* 464 $(M)^+$; ^{1}H NMR (DMSO-d_6) δ 3.69 (d, J = 6.0 Hz, 2H, CH_2O), 4.40 (d, J = 6.0 Hz, 2H, CH_2N), 5.58−5.77 (m, 2H, CH=CH), 6.33 (br s, 2H, NH_2), 7.23−7.39 (m, 15H, Ph), 7.48 (s, 1H, H-8), 10.51 (br s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ 39.50 (CH_2N), 59.83 (CH_2O), 86.51 (*C*Ph_3), 116.49 (C-5), 126.30, 130.29 (CH=CH), 127.11, 127.99, 128.20, 143.70 (Ph), 150.91 (C-4), 153.48 (C-2), 156.77 (C-6). Anal. for $C_{28}H_{25}N_5O_2$: C, H, N.

5-Methyl-1-[(*Z*)-4-(triphenylmethoxy)-2-butenyl]cytosine (25). A suspension of 1,2,4-triazole (189 mg, 2.74 mmol) and $POCl_3$ (0.15 mL, 1.80 mmol) in dry CH_3CN (2 mL) was stirred at 0 °C for 5 min. Then Et_3N (0.78 mL, 5.60 mmol) was slowly added. The resulting mixture was stirred at 0 °C for 1 h, and then a solution of **10** (150 mg, 0.34 mmol) in dry CH_3CN (1 mL) was added. The mixture was stirred at room temperature for 18 h and then filtered. The filtrate was diluted with EtOAc (20 mL) and washed with aqueuos $NaHCO_3$ (10 mL). The aqueous phase was further extracted with EtOAc (2 × 15 mL). The combined organic extracts were dried on anhydrous Na_2SO_4, filtered, evaporated, and coevaporated with dioxane. The residual oil was dissolved in dioxane (2 mL) and treated with NH_4OH (2 mL) for 1 h. Volatiles were removed, and the residue was purified by flash column chromatography (hexane/EtOAc, 2:1). The fractions containing **25** were further purified by CCTLC in the Chromatotron (EtOAc/MeOH, 10:1) to yield 32 mg (22%) of **25**: mp 167−171 °C; MS (ES, positive mode), *m*/*z* 460 $(M + Na)^+$; ^{1}H NMR ($CDCl_3$) δ 1.80 (s, 3H, 5-CH_3), 3.71 (d, J = 6.6 Hz, 2H, CH_2O), 4.24 (d, J = 7.1 Hz, 2H, CH_2N), 5.60, 5.90 (m, 2H, CH=CH), 6.92 (s, 1H, H-6), 7.17−7.46 (m, 15H, Ph), 8.13 (br s, 2H, NH_2); ^{13}C NMR ($CDCl_3$) δ 12.93 (5-CH_3), 45.48 (CH_2N), 59.58 (CH_2O), 87.14 (*C*Ph_3), 101.63 (C-5), 127.09, 130.69 (CH=CH), 127.13, 127.92, 128.52, 142.18 (Ph), 143.76 (C-6), 156.64 (C-2), 165.47 (C-4). Anal. for $C_{28}H_{27}N_3O_2$: C, H, N.

1-[(*Z*)-4-(Triphenylmethoxy)-2-butenyl]-5,6-dihydro-

thymine (26). To a solution of **10** (150 mg, 0.34 mmol) in dry THF (2 mL) at 0 °C and under Ar atmosphere was added a 1.2 M solution of DibalH in hexane (0.7 mL, 3.40 mmol). The mixture was allowed to reach room temperature and stirred overnight. The reaction was quenched by the addition of MeOH (2 mL) and evaporated. The residue was extracted several times with hot MeOH and filtered. The filtrate was evaporated and then purified by CCTLC in the Chromatotron (hexane/EtOAc, 1:1) to yield 81 mg (54%) of **26** as an amorphous solid: MS (ES, positive mode), *m*/*z* 463 $(M + Na)^+$; ^{1}H NMR ($CDCl_3$) δ 1.58 (d, $J = 7.0$ Hz, 3H, 5-CH_3), 2.57 (m, 1H, H-5), 2.94 (pt, 1H, H-6), 3.17 (dd, $J = 12.4$, 6.2 Hz, 1H, H-6), 3.67 (d, $J = 6.4$ Hz, 2H, CH_2O), 3.87 (d, $J = 5.7$ Hz, 2H, CH_2N), 5.46, 5.94 (m, 2H, CH=CH), 7.18−7.45 (m, 15H, Ph), 8.34 (br s, 1H, 3-NH); ^{13}C NMR ($CDCl_3$) δ 12.67 (5-CH_3), 35.35 (C-5), 43.47 (CH_2N), 47.74 (C-6), 59.51 (CH_2O), 87.04 (*C*Ph_3), 126.31, 131.26 (CH=CH), 127.14, 127.89, 128.56, 143.82 (Ph), 152.35 (C-2), 172.42 (C-4). Anal. for $C_{28}H_{28}N_2O_3$: C, H, N.

3-Methyl-1-[(*Z*)-4-(triphenylmethoxy)-2-butenyl]thymine (27). A suspension containing compound **10** (150 mg, 0.34 mmol) and K_2CO_3 (24 mg, 0.17 mmol) in acetone (3 mL) was stirred at room temperature for 10 min. Then CH_3I (0.032 mL, 0.51 mmol) was added, and the mixture was stirred at room temperature for 48 h. Then it was diluted with CH_2Cl_2 (20 mL) and aqueous $NaHCO_3$ (10 mL). The aqueous phase was further extracted with CH_2Cl_2 (2 × 10 mL). The combined organic extracts were dried on anhydrous Na_2SO_4, filtered, and evaporated. The residue was purified by CCTLC in the Chromatotron (hexane/EtOAc, 1:1) to yield 143 mg (93%) of **27**: mp 133−135 °C; MS (ES, positive mode), *m*/*z* 475 $(M + Na)^+$; ^{1}H NMR ($CDCl_3$) δ 1.84 (s, 3H, 5-CH_3), 3.32 (s, 3H, 3-NCH_3), 3.75 (d, $J = 5.3$ Hz, 2H, CH_2O), 4.22 (d, $J = 5.9$ Hz, 2H, CH_2N), 5.55, 5.92 (m, 2H, CH=CH), 6.84 (s, 1H, H-6), 7.20−7.34 (m, 15H, Ph); ^{13}C NMR ($CDCl_3$) δ 12.99 (5-CH_3), 27.98 (3-NCH_3), 45.42 (CH_2N), 59.69 (CH_2O), 87.32 (*C*Ph_3), 109.89 (C-5), 126.11, 131.51 (CH=CH), 127.20, 127.96, 128.59, 143.78 (Ph), 137.39 (C-6), 151.61 (C-2), 163.88 (C-4). Anal. for $C_{29}H_{28}N_2O_3$: C, H, N.

General Procedure for the Esterification of the Trityl Ether 10. Synthesis of 28—32. To a solution of **10** (150 mg, 0.34 mmol) in CH_2Cl_2 (14 mL) at 0 °C was added a Dowex resin 50WX4 (H^+ form) (34 mmol) and the corresponding acyl chloride (17 mmol). The mixture was allowed to reach room temperature and stirred for 24 h. The reaction mixture was diluted with CH_2Cl_2 (50 mL) and filtered. The filtrate was washed with a solution of aqueous $NaHCO_3$ (15 mL). The organic phase was dried on anhydrous Na_2SO_4, filtered, and evaporated. The residue was purified by CCTLC in the Chromatotron (hexane/EtOAc, 1:1).

4-(Thymin-1-yl)but-2-enyl-2,2-dimethyl propionate (28) was prepared according to the general procedure by reaction of **10** with pivaloyl chloride: yield, 66%; mp 168−171 °C; MS (ES, positive mode), *m*/*z* 303 $(M + Na)^+$; ^{1}H NMR ($CDCl_3$) δ 1.19 [s, 9H, $(CH_3)_3C$)], 1.89 (s, 3H, 5-CH_3), 4.46 (d, $J = 6.6$ Hz, 2H, CH_2N), 4.67 (d, $J = 6.6$ Hz, 2H, CH_2O), 5.64, 5.72 (m, 2H, CH=CH), 7.14 (s, 1H, H-6), 8.60 (br s, 1H, 3-NH); ^{13}C NMR ($CDCl_3$) δ 12.28 (5-CH_3), 27.09 [($CH_3)_3C$)], 38.71 [$(CH_3)_3C$)], 44.18 (CH_2N), 59.26 (CH_2O), 111.07 (C-5), 128.01, 128.61 (CH=CH), 139.96 (C-6), 150.08 (C-2), 164.20 (C-4), 178.45 (CO). Anal. for $C_{14}H_{20}N_2O_4$: C, H, N.

4-(Thymin-1-yl)but-2-enyl-3,3-dimethyl butyrate (29) was prepared according to the general procedure by reaction of **10** with *tert*-butylacetyl chloride: yield, 56%; mp 128−131 °C; MS (ES, positive mode), *m*/*z* 295 $(M + 1)^+$, 317 $(M + Na)^+$; ^{1}H NMR ($CDCl_3$) δ 1.04 [s, 9H, ($CH_3)_3$C], 1.90 (s, 3H, 5-CH_3), 2.23 (s, 2H, CH_2CO), 4.50 (d, $J = 6.8$ Hz, 2H, CH_2N), 4.70 (d, $J = 7.0$ Hz, 2H, CH_2O), 5.64, 5.79 (m, 2H, CH=CH), 7.17 (s, 1H, H-6), 8.94 (br s, 1H, 3-NH); ^{13}C NMR ($CDCl_3$) δ 12.32 (5-CH_3), 29.69 [($CH_3)_3$C], 30.94 [$(CH_3)_3C$)], 44.29 (CH_2CO), 47.84 (CH_2N), 58.94 (CH_2O), 111.21 (C-5), 128.16, 128.65 (CH=CH), 140.06 (C-6), 150.92 (C-2), 164.11 (C-4), 172.26 (CO). Anal. for $C_{15}H_{22}N_2O_4$: C, H, N.

4-(Thymin-1-yl)but-2-enyl hexanoate (30) was prepared according to the general procedure by reaction of **10** with hexanoyl chloride: yield, 65%; mp 93−95 °C; MS (ES, positive mode), *m*/*z* 295 $(M + 1)^+$, 317 $(M + Na)^+$; ^{1}H NMR ($CDCl_3$) δ 0.86 (t, $J = 6.6$ Hz, 3H, CH_3), 1.22−1.39 (m, 4H, CH_2), 1.61 (q, 4H, CH_2), 1.88 (s, 3H, 5-CH_3), 2.30 (t, $J = 6.3$ Hz, 2H, CH_2-CO), 4.44 (d, $J = 6.6$ Hz, 2H, CH_2N), 4.68 (d, $J = 6.8$ Hz, 2H, CH_2O), 5.60, 5.76 (m, 2H, CH=CH), 7.09 (s, 1H, H-6), 9.08 (br s, 1H, 3-NH); ^{13}C NMR ($CDCl_3$) δ 12.28, 13.85 (5-CH_3, CH_3), 22.26, 24.55, 31.22, 34.12 (CH_2), 44.26 (CH_2N), 59.16 (CH_2O), 111.09 (C-5), 127.97, 128.60 (CH=CH), 139.87 (C-6), 150.89 (C-2), 164.15 (C-4), 173.67 (CO). Anal. for $C_{15}H_{22}N_2O_4$: C, H, N.

4-(Thymin-1-yl)but-2-enyl laureate (31) was prepared according to the general procedure by reaction of **10** with lauroyl chloride and purified by CCTLC in the Chromatotron (hexane/EtOAc, 1:1): yield, 49%; mp 62−65 °C; MS (ES, positive mode), *m*/*z* 379 $(M + 1)^+$, 401 $(M + Na)^+$; ^{1}H NMR ($CDCl_3$) δ 0.85 (t, 3H, $J = 6.8$ Hz, CH_3), 1.22−1.37 (m, 16H, CH_2), 1.59 (q, $J = 7.1$ Hz, 2H, CH_2CH_2CO), 1.88 (s, 3H, 5-CH_3), 2.30 (t, $J = 7.3$ Hz, 2H, CH_2CO), 4.45 (d, $J = 6.8$ Hz, 2H, CH_2N), 4.68 (d, $J = 6.6$ Hz, 2H, CH_2O), 5.58−5.79 (m, 2H, CH=CH), 7.10 (s, 1H, H-6), 8.87 (br s, 1H, 3-NH). Anal. for $C_{21}H_{34}N_2O_4$: C, H, N.

4-(Thymin-1-yl)but-2-enyl-diphenylacetate (32) was prepared according to the general procedure by reaction of **10** with diphenylacetyl chloride: yield, 81%; mp 105−110 °C; MS (ES, positive mode), *m*/*z* 391 $(M + 1)^+$, 413 $(M + Na)^+$; ^{1}H NMR ($CDCl_3$) δ 1.85 (s, 3H, 5-CH_3), 4.43 (d, $J = 6.6$ Hz, 2H, CH_2N), 4.80 (d, $J = 6.8$ Hz, 2H, CH_2O), 5.07 [s, 1H, $CH(Ph)_2$], 5.60−5.75 (m, 2H, CH=CH), 7.05 (s, 1H, H-6), 7.28−7.40 (m, 10H, Ph), 9.32 (br s, 1H, 3-NH); ^{13}C NMR ($CDCl_3$) δ 12.20 (5-CH_3), 44.28 (CH_2N), 56.89 (*C*Ph_2), 60.06 (CH_2O), 111.02 (C-5), 127.46, 127.83 (CH=CH), 128.50, 128.59, 128.69, 138.26 (Ph), 139.98 (C-6), 150.70 (C-2), 164.02 (C-4), 172.49 (CO). Anal. for $C_{23}H_{22}N_2O_4$: C, H, N.

1-[(*Z*)-4-Hydroxy-2-butenyl]thymine (33). A solution of **10** (150 mg, 0.34 mmol) in MeOH (12 mL) was treated with a Dowex resin 50WX4 (H^+ form) (34 mmol) at room temperature overnight. The mixture was diluted by the addition of MeOH (50 mL) and filtered. The filtrate was evaporated and purified in the Chromatotron CCTLC (CH_2Cl_2/MeOH, 15:1) to yield 65 mg (98% yield) of **33** as a white solid: mp 166−169 °C (lit.[17] mp 163−166 °C); Anal. for $C_9H_{12}N_2O_3$: C, H, N.

4-(Thymin-1-yl)but-2-enyl benzoate (34). To a solution of **33** (50 mg, 0.25 mmol) in dry CH_2Cl_2 (3 mL) and pyridine (0.5 mL) was added benzoyl chloride (0.035 mL, 0.30 mmol). The mixture was stirred at room temperature for 6 h. Then it was treated with CH_2Cl_2 (20 mL) and 1 N HCl solution (10 mL). The organic phase was washed with H_2O, dried on anhydrous Na_2SO_4, filtered, and evaporated. The residue was purified in an SPE cartridge (silica) eluting with CH_2Cl_2/EtOAc to yield 40 mg (53%) of **34** as a white solid: MS (ES, positive mode), *m*/*z* 287 $(M + 1)^+$, 309 $(M + Na)^+$; ^{1}H NMR was identical to that reported.[29] Anal. for $C_{16}H_{16}N_2O_4$: C, H, N.

1-[(*Z*)-4-(Benzyloxy)-2-butenyl]thymine (36). Following a procedure analogous to that described for the Mitsunobu condensation of alcohols with N^3-BzT, alcohol **35**[30] (100 mg, 0.56 mmol) reacted with N^3-BzT (258 mg, 1.1 mmol) to afford **36** (94 mg, 59%) as a syrup: ^{1}H NMR ($CDCl_3$) δ 1.80 (s, 3H, 5-CH_3), 3.14 (d, $J = 5.5$ Hz, 2H, CH_2O), 4.37 (d, $J = 7.1$ Hz, 2H, CH_2N), 4.53 (s, 2H, CH_2Ph), 5.59, 5.80 (m, 2H, CH=CH), 6.96 (s, 1H, H-6), 7.30 (m, 5H, Ph), 9.29 (br s, 1H, 3-NH); ^{13}C NMR ($CDCl_3$) δ 12.18 (5-CH_3), 44.48 (CH_2N), 65.61 (CH_2O), 72.88 (*C*H_2Ph), 110.89 (C-5), 126.57, 131.26 (CH=CH), 127.86, 127.92, 128.50, 137.67 (Ph), 139.70 (C-6), 150.91 (C-2), 164.17 (C-4). Anal. for $C_{16}H_{18}N_2O_3$: C, H, N.

Biological Procedures. A. TK Assay Using [*methyl*-^{3}H]-dThd as the Substrate. The preparation and purification of recombinant HSV-1 TK, HSV-1 (A167Y) TK, and TK-2 has been described previously.[6,10,12,31,32] The activity of purified recombinant HSV-1 TK and mitochondrial TK-2 was assayed in a 50-μL reaction mixture containing 50 mM Tris-HCl (pH 8.0), 2.5 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM CHAPS, 3 mg/mL BSA, 2.5 mM ATP, 2 μM [*methyl*-^{3}H]dTd or 1.6 μM [2,8-^{3}H]GCV (1 μCi), and enzyme (CHAPS was replaced by 10

mM NaF in the reaction mixtures for HSV-1 TK). The samples were incubated at 37 °C for 30 min in the presence or absence of different concentrations of the test compounds. Due to the poor solubility of the compounds in water, stock solutions were made in pure DMSO. Appropriate dilutions of the test compounds were performed in 100% DMSO. When added to the enzyme reaction mixture, constant volumes of each of the compound dilutions were used to achieve a DMSO concentration of 10% in all samples, including the enzyme controls. At this DMSO concentration the enzyme catalytic activity was not significantly affected. Precipitation of the (lipophilic) test compounds is avoided for most of the compounds at 50 μM in the presence of 10% DMSO. Therefore, compound concentrations >50 μM may not be accurate due to insolubility, even in the presence of 10% DMSO. Aliquots of 45 μL of the reaction mixtures were spotted on Whatman DE-81 filter paper disks. The filters were washed three times for 5 min each in 1 mM ammonium formate, once for 1 min in water, and once for 5 min in ethanol. The radioactivity was determined by scintillation counting. The IC_{50} was defined as the drug concentration required to inhibit the enzyme activity by 50%. The tested compound dilutions were 5-fold. The IC_{50} values were derived from an extrapolation of the data obtained in the presence of an immediately higher and immediately lower concentration than the IC_{50} value.

The following formula for the calculation of IC_{50} was used:

$$IC_{50} = A - \frac{50\% - \%\text{ cell growth at } A}{\%\text{ cell growth at } B - \%\text{ cell growth at } A} \times [A - B] \quad (1)$$

In eq 1 A is the higher drug concentration and B is the lower drug concentration

Effect of dThd and HSV-1 TK Inhibitors on the Cytostatic Activity of GCV and BVaraU in Human Osteosarcoma Cells Transduced with the HSV-1 TK Gene. The cytostatic activity of GCV and BVaraU against HSV-1 TK gene-transduced human osteosarcoma cells was evaluated as follows: 10^4 OST TK^-/HSV-1 TK^+ cells/well were plated in 96-well microtiter plates and subsequently incubated in the presence of 5-fold dilutions of the test compounds GCV and BVaraU in the absence or presence of 20 μM of compounds **10** or **12** (in the presence of 1% DMSO, which is not toxic to the cell cultures) or 50 μM dThd (in the absence of DMSO). After 3 days, the number of cells was counted in a Coulter counter (Coulter Electronics Ltd., Harpenden, U.K.). The IC_{50} was defined as the drug concentration required to inhibit tumor cell proliferation by 50%.

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