Synthesis, Kinetics, and Molecular Docking of Novel 9-(2-Hydroxypropyl)purine Nucleoside Analogs as Ligands of Herpesviral Thymidine Kinases

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Dedicated to *Dieter Seebach* on the occasion of his 65th birthday

In the context of broadening the knowledge on substrate specificity of *Herpes simplex* virus type 1 thymidine kinase (HSV-1 TK) and *Varicella-Zoster* virus thymidine kinase (VZV TK), new derivatives of 9-(2-hydroxypropyl)-substituted adenine, chloropurine, hypoxanthine, guanine, thiopurine, and (methylsulfanyl)-purine were synthesized and subjected to *in vitro* phosphorylation and binding affinity assays. The interactions between the compounds and the crystallographically determined active site residues of HSV-1 TK have been studied by molecular modeling with the Lamarckian genetic algorithm of docking program AutoDock 3.0. All compounds mentioned bind to both enzymes in the low mM to sub-mM range, comparable to binding affinities of existing prodrugs. Findings from the docking procedure indicate multiple binding modes for all of the compounds and are in accordance with the results of phosphorylation and binding-affinity studies. Furthermore, the studies reveal that hypoxanthine derivatives represent a new class of TK substrates and thiopurine derivatives a new class of TK inhibitors.

Introduction. – Herpesviral thymidine kinases belong to the pyrimidine salvage pathway and phosphorylate thymidine (dT), leading to thymidine monophosphate (dTMP). *Herpes simplex* virus type 1 thymidine kinase (HSV-1 TK) and *Varicella-Zoster* thymidine kinase (VZV TK) play a key role in pathogenesis and reactivation [1–3]. Aciclovir (9-[(2-hydroxyethoxy)methyl]guanine; ACV), penciclovir (9-[4-hydroxy-3-(hydroxymethyl)butyl]guanine; PCV), and other prodrugs are therapeutic compounds, which interfere with acute herpes-virus infections [4]. After being activated exclusively by the viral TK, these molecules act as fraudulent substrates of DNA-polymerases, blocking DNA replication by dead-end complexes with the viral DNA. The molecular basis of the therapy, which uses viral TK as selectivity filter, is the difference in substrate specificity between the herpesviral TK and the human cellular isoenzyme. Furthermore, HSV-1 TK and VZV TK in combination with ganciclovir (9-{[2-hydroxy-1-(hydroxymethyl)ethoxy]methyl}guanine; GCV) and bromovinyldeoxyuridine (BVDU), respectively, are more recently used as suicide enzymes in gene

[1]) Equally contributed to the work.

therapy of cancer [5][6–8], as well as a control of the graft *vs.* host disease (GvHD) in the context of the stem-cell transplantation [9]. HSV-1 TK is also used in combination with molecules labeled with positron-emitting radioisotopes as an *in situ* reporter, which allows monitoring suicide gene therapy, *e.g.*, the expression level of the suicide gene product and its location [10–12]. The fact that viral TKs play a role in establishing latent infection and recurrent disease of herpes viruses leads to another therapeutical approach. TK Inhibitors may play a beneficial role in prevention and reduction of virus reactivation [13][14].

Both HSV-1 TK and VZV TK provide useful properties for the different therapeutic strategies. They share many structural and biochemical features of all thymidine kinases of the Herpesviridae. However, some important differences exist in the substrate specificities of these enzymes. For example, ACV is readily phosphorylated by HSV-1 TK, but poorly phosphorylated by VZV TK [15]. The question remains whether the difference in substrate acceptance between HSV-1 TK and VZV TK is limited to the cases known in the literature [16], or if it covers a wider range of the chemical space defined around the purine scaffold. Furthermore, the issue of the multiple-binding mode, observed by X-ray crystallography with purine derivatives with low binding affinity towards HSV-1 TK [17][18], is addressed.

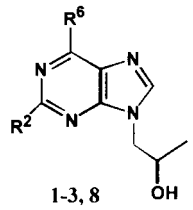
These facts have prompted us to design and synthesize compounds with varying purine moieties, namely 9-(2-hydroxypropyl)-substituted adenine (**1**), chloropurines (**2** and **3**), hypoxanthine (**4**), guanine (**5**), thiopurine (**6**, **7**), and (methylsulfanyl)purine (**8**) derivatives (*Scheme*). To explore the substrate acceptance of viral TKs and to widen the knowledge applicable to different treatments, the new compounds were subjected to *in vitro* phosphorylation tests and affinity measurements with HSV-1 and VZV TK. Their structure-activity relationship analysis with respect to binding and function (*e.g.*, substrate or inhibition) were performed by molecular docking to the active site of the crystal structure of HSV-1 TK [17]. The presented work indicates that the selected molecular-docking procedure enables us to study alternate modes of binding and, thus, mapping of the active site.

Results and Discussion. – *Chemistry.* New *N*(9)-substituted acyclic purine nucleoside analogs **1**–**8** (*Scheme*, *a*) were prepared by a route involving alkylation of *C*(6)- and/or *C*(2)-substituted purine derivatives with propylene carbonate in the presence of a catalytic amount of NaOH according to the procedure for preparation of 9-(2-hydroxyethyl)adenine (*Scheme*, *b*) [19]. Conversion of the 6-Cl-substituted compounds **2** and **3** into the corresponding keto derivatives **4** and **5** was accomplished by acidic hydrolysis (*Scheme*, *b*) by the analogous procedure reported for the preparation of 9-(4-hydroxy-3-(hydroxymethyl)-2-methylbutyl)guanine [20]. The thio derivatives **6** and **7** were obtained by reaction of the 6-chloropurine derivatives **2** and **3** with thiourea in absolute EtOH and in the presence of a catalytic amount of HCOOH [21]. The synthesis of 9-(2-hydroxypropyl)-6-(methylsulfanyl)purine (**8**) was accomplished by methylation of the thio derivative **6** [22].

^{1}H- and ^{13}C-NMR Spectra. Assignment of ^{1}H- and ^{13}C-NMR spectra was performed on the basis of chemical shifts, signal intensities, magnitude and multiplicity of C,H spin-spin coupling constants, as well as connectivity in NOESY and COSY spectra. The ^{1}H- and ^{13}C-NMR data of **1**–**8** are collected in *Tables 1* and *2*, respectively.

Scheme. *9-(2-Hydroxypropyl)purine Nucleoside Analogs and the Procedure for Their Preparation*

a)



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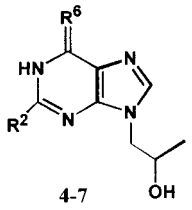

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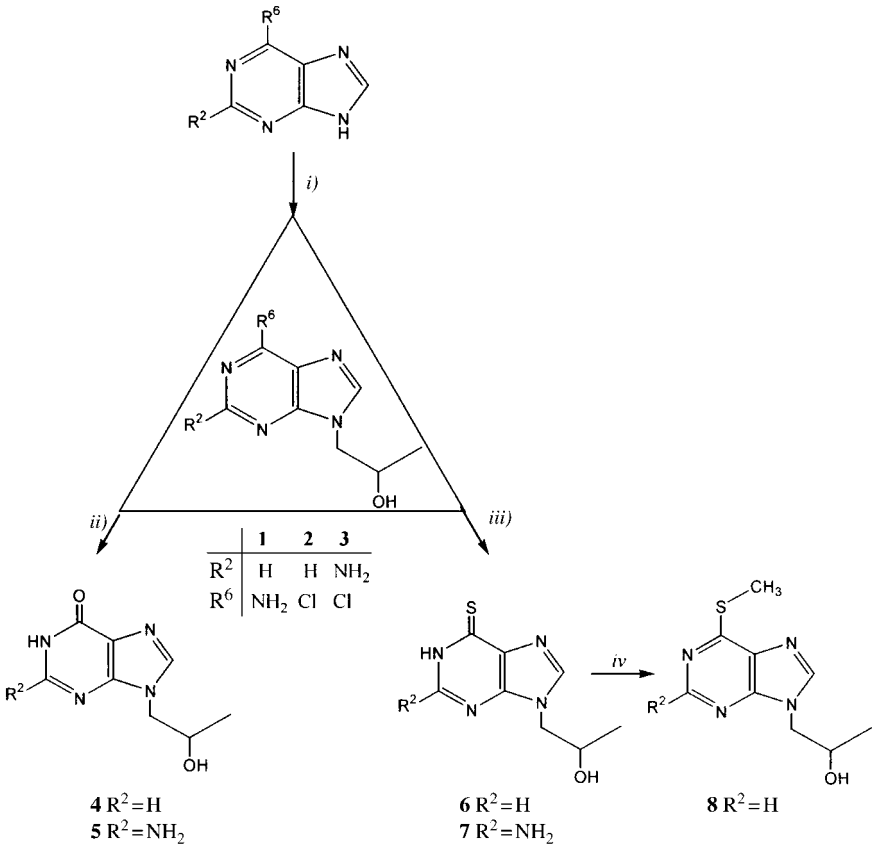
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
R^2	H	H	NH_2	H	NH_2	H	NH_2	H
R^6	NH_2	Cl	Cl	O	O	S	S	MeS

b)



	1	**2**	**3**
R^2	H	H	NH_2
R^6	NH_2	Cl	Cl

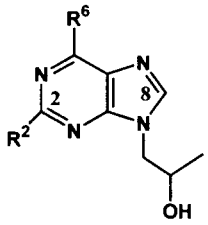
4 R^2=H
5 R^2=NH_2

6 R^2=H
7 R^2=NH_2

8 R^2=H

i) Propylene carbonate, NaOH, dry DMF, reflux, 10 h. *ii*) 2.5M HCl, reflux, 2 h. *iii*) Thiourea, HCOOH/anh. EtOH, reflux, 1 h. *iv*) NaOH, dimethyl sulfate, H_2O, r.t., 1.5 h.

Table 1. *^{1}H-NMR Chemical Shifts* (δ/ppm)[a]) *and H,H Coupling Constants* (J/Hz)[b]) *for Compounds* **1**–**8**



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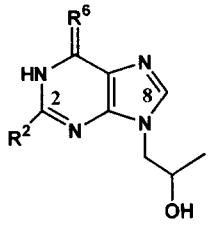

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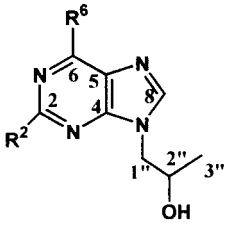
Compound	H–C(2)	H–C(8)	NH_2	NH	CH_2N	CH	CHO*H*	Me
1	8.15	8.06	7.26		4.13–3.99	4.03	5.06	1.07
	(*s*, 1 H)	(s, 1 H)	(*s*, 2 H)		(*m*, 2 H)	(*m*, 1 H)	(*d*, *J* = 3.5, 1 H)	(*d*, *J* = 5.67, 3 H)
2	8.77	8.61			4.31–4.15	4.09	5.07	1.10
	(*s*, 1 H)	(*s*, 1 H)			(*m*, 2 H)	(*m*, 1 H)	(*d*, *J* = 4.7, 1 H)	(*d*, *J* = 5.9)
3		8.27	7.0		4.30–4.13	4.01	5.36	1.24
		(*s*, 1 H)	(*s*, 2 H)		(*m*, 2 H)	(*m*, 1 H)	(*d*, *J* = 4.9, 1 H)	(*d*, *J* = 5.9)
4	8.04	8.01		8.4	4.14–4.04	4.01	6.40	1.05
	(*s*, 1 H)	(*s*, 1 H)		(*s*, 1 H)	(*m*, 2 H)	(*m*, 1 H)	(br., 1 H)	(*d*, *J* = 5.9)
5		7.61	6.51	10.7	3.94–3.85	3.81	5.03	1.07
		(*s*, 1 H)	(*s*, 2 H)	(*s*, 1 H)	(*m*, 2 H)	(*m*, 1 H)	(*d*, *J* = 4.4, 1 H)	(*d*, *J* = 5.9)
6	8.21	8.21		13.7	4.17–4.02	4.02	5.07	1.07
	(*s*, 1 H)	(*s*, 1 H)		(*s*, 1 H)	(*m*, 2 H)	(*m*, 1 H)	(br., 1 H)	(*d*, *J* = 5.9)
7		7.81	6.80	11.9	3.93–3.84	3.81	5.05	1.05
		(*s*, 1 H)	(*s*, 2 H)	(*s*, 1 H)	(*m*, 2 H)	(*m*, 1 H)	(*d*, *J* = 4.4, 1 H)	(*d*, *J* = 5.9)
8	8.72	8.37			4.24–4.10	4.06	5.05	1.08
	(*s*, 1 H)	(*s*, 1 H)			(*m*, 2 H)	(*m*, 1 H)	(*d*, *J* = 3.1, 1 H)	(*d*, *J* = 5.3)

[a]) (D_6)DMSO Solutions, chemical shifts referred to TMS. Multiplicity and number of H-atoms are given in parentheses.
[b]) Digital resolution ±0.25 Hz.

Analysis of 1D and 2D ^{1}H- and ^{13}C-NMR spectra has shown that alkylation of the adenine, 6-chloropurine, 2-amino-6-chloropurine, hypoxanthine, guanine, purine-6(1*H*)thione, 2-aminopurine-6(1*H*)-thione, and 6-(methylsulfanyl)purine ring by the 2-hydroxypropyl aliphatic chain took place at N(9). This was concluded from the chemical-shifts pattern of the purine moiety in both ^{1}H- and ^{13}C-NMR spectra, which are consistent with those observed for other *N*(9)-substituted purine nucleoside analogs [23–25]. Thus, in **1**–**8**, H–C(2) is more deshielded (8.77–8.04 ppm) than H–C(8) (8.61–7.61), and the same is valid for C-atoms C(2) (162.0–145.05 ppm) and C(8) (148.40–138.49 ppm). In addition, in NOESY spectra, cross-peaks between *N*-CH_2 H-atoms and H–C(8) were observed, which would not be possible in the case of *N*(3) derivatives due to a much greater spatial distance between the corresponding H-atoms.

The *N*(9) side-chain H,H coupling patterns in **1**–**8** are: two *multiplets* (4.31–3.84 ppm) and (4.09–3.81 ppm) corresponding to *N*-CH_2 and *O*-CH H-atoms, respectively, *doublet* (5.36–5.03 ppm) or broad signal (6.4 and 5.07 ppm), corresponding to the OH group, and *doublet* (1.24–1.04 ppm) for the Me group. In all compounds investigated, the nonequivalence of *N*–CH_2 H-atoms exists. The *N*–CH_2 H-atoms

Table 2. *^{13}C-NMR Chemical Shifts* (δ/ppm)[a]) *for Compounds* **1**–**8**



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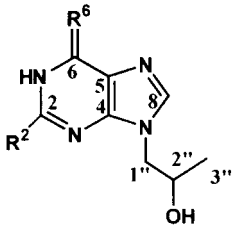

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Compound	C(2)	C(4)	C(5)	C(6)	C(8)	C(1″)	C(2″)	C(3″)
1	152.58	150.01	118.78	156.23	141.79	50.24	64.78	20.90
2	151.64	149.09	130.94	152.52	148.40	50.98	64.50	20.78
3	162.00	151.38	125.60	156.48	145.72	52.07	66.48	21.96
4	145.68	148.91	123.93	157.10	141.31	50.50	64.90	20.89
5	153.80	151.61	116.55	157.21	138.49	50.03	64.74	20.98
6	145.05	144.65	135.50	175.99	144.03	50.61	64.77	20.81
7	153.23	148.37	128.38	175.00	141.65	50.09	64.59	20.95
8[b])	151.62	148.08	130.95	161.62	143.62	51.65	66.06	20.45

[a]) (D_6)DMSO Solutions, chemical shifts referred to TMS. Digital resolution ±1.18 Hz. [b]) $CDCl_3$ solution. Chemical shifts for *Me*S at 11.51 ppm.

(4.31–3.84 ppm) are more deshielded than the *O*–CH H-atoms (4.09–3.81 ppm). All compounds, except for **8**, which was not amenable to the chosen HPLC conditions, eluted as single peaks.

Phosphorylation Screening by HPLC. The extent of phosphorylation of the studied 9-(2-hydroxypropyl) derivatives was measured by exposing the compound to recombinantly-expressed HSV-1 or VZV TK in the presence of ATP and Mg^{2+} (see *Exper. Part*), and subsequent separation and analysis of the reaction mixture by ion-pair chromatography [26]. The mean values of the calculated ADP/ATP ratios and relative standard deviation of the three different experiments measured as triplicates with HSV-1 TK and VZV TK are reported in *Fig. 1, a* and *b*, respectively. Despite of the slight formation of ADP in all experiments, the amount of released ADP significantly differed between the phosphorylation assays and the blanks (*Fig. 1*). Comparison of HSV-1 TK and VZV TK reveals a higher ADP/ATP ratio for VZV TK as a consequence of a difference in stability between the two enzymes over a longer period of time [27]. Therefore, HPLC results are compared relatively to each other.

The 6-(methylsulfanyl)purine derivative **8** could not be assayed with HPLC; therefore, its phosphorylation was determined with a pyruvate-kinase/lactate-dehydrogenase-coupled UV-spectrophotometric assay [28][29] (see *Exper. Part*), and results are presented in *Fig. 2*.

HSV-1 TK phosphorylates all compounds except the purine-6-thione derivative **6** (*Fig. 1*). The adenine, 2-aminopurine-6-thione, and 6-(methylsulfanyl)purine derivatives, **1**, **7** and **8**, respectively, were poorly phosphorylated, while compounds **2**–**5** were increasingly transformed into their monophosphate derivatives (*Fig. 1,a* and *Fig. 2*).

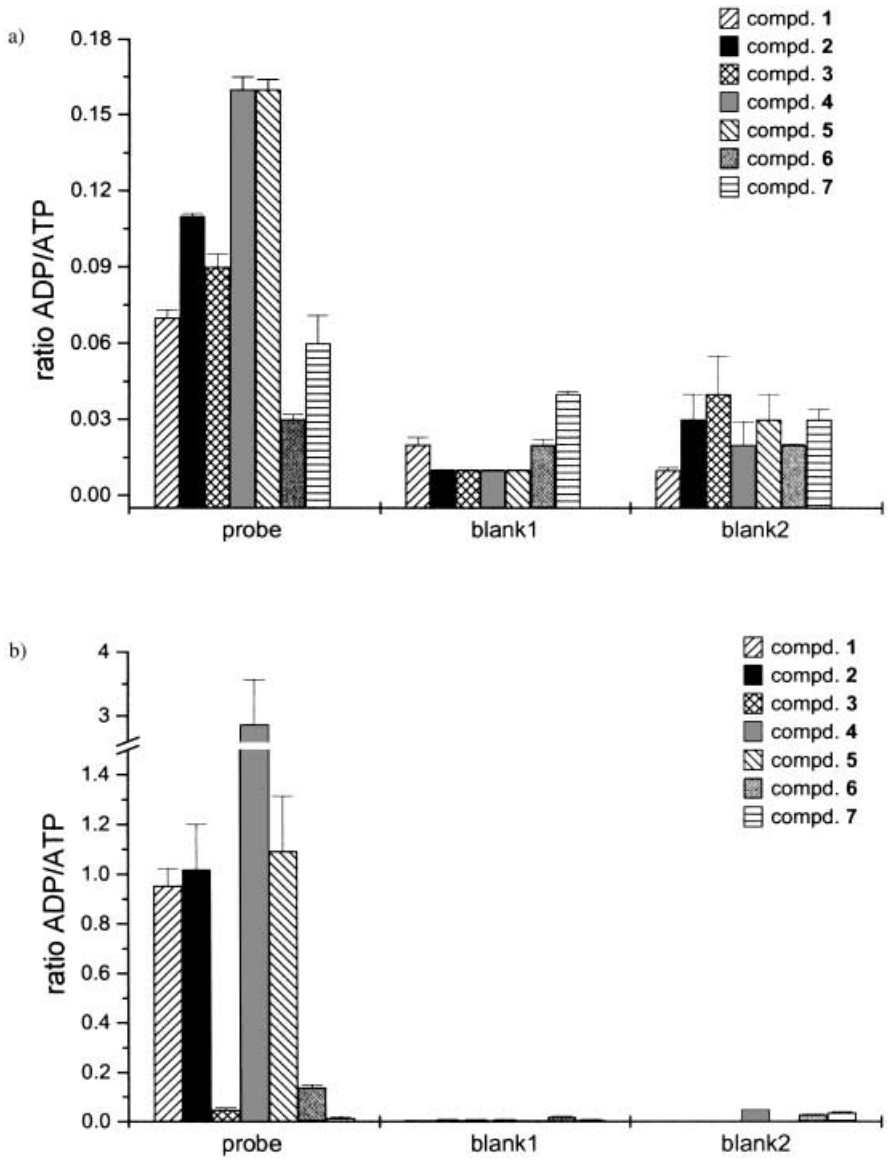

Fig. 1. *Histogram of the ratio between area of ADP and area of ATP resulting from the different experiments of compounds* **1**–**7** *using HSV-1 TK* (a) *and VZV TK* (b). Blank1 (same reaction mixture as for probe but without TK) and blank2 (same reaction mixture as for probe except compound) experiments were performed to monitor spontaneous hydrolysis of ATP to ADP under the experimental conditions. The negative control was done with a compound not exhibiting the OH group acting as a phosphate acceptor (data not shown). The ordinate is interrupted for clarity reasons. The data of compound **4** may be slightly overestimated because only partial separation (base-valley) of the ADP and compound peak could be achieved. The relative standard deviation is given as vertical bars. The experiments have been performed in triplicates.

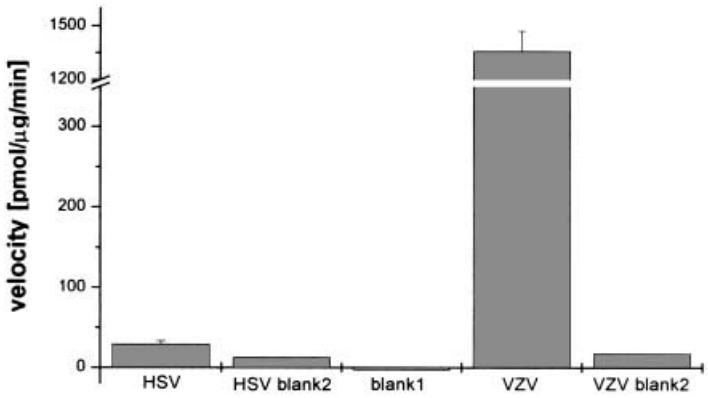

Fig. 2. *Histogram of the ADP formation velocity with HSV-1 and VZV TK at 6 mM concentration of the compound* **8**. Equal amounts of enzyme (4 µg) were used. The presented data are mean values of three independent measurements and take into account the spontaneous hydrolysis of ATP to ADP. The relative standard deviation is given as vertical bars. The ordinate is interrupted for clarity reasons. The blank reactions have been performed in analogy to HPLC and contained no enzyme (blank1) or no compound (HSV blank2; VZV blank2).

Contrary to HSV-1 TK, VZV TK failed to phosphorylate compounds **3** and **7** (*Fig. 1, b*). For compound **6**, only a very slight phosphorylation by VZV TK was detected, while significant phosphorylation was observed for compounds **1**, **2**, **4**, and **5**. The photometric assay reveals significant phosphorylation by VZV TK of the 6-(methylsulfanyl)purine derivative (**8**; *Fig. 2*).

Both TKs are able to phosphorylate compound **2**, implying that the Cl substituent might take over the role of the carbonyl O-atom at C(6) in the corresponding purine analog. Both enzymes readily adopted the hypoxanthine derivative **4** as a substrate. Not unexpectedly, compound **5**, similar to ACV, was phosphorylated by both TKs; however, less by VZV TK. The 6-thiopurine acyclic analog **6** was poorly accepted as a substrate by both enzymes. Compound **7** was phosphorylated to some extent by HSV-1 TK, but not by VZV TK. The comparison of the phosphorylation pattern of compounds **2** and **3**, **4** and **5**, as well as **6** and **7**, reveals that, in contrast to HSV-1 TK, VZV TK is severely impaired in its function when an NH_2 group is present at C(2) of the purine moiety.

Binding-Affinity Studies. To evaluate the binding affinities of the purine derivatives, competition studies were performed with isotopically labeled thymidine and the corresponding compounds **1**–**8** as the competitive ligands. Initial velocity (V_i) was determined by monitoring the increase of the labeled dTMP over time in the absence and presence of various concentrations of the compound. Those experiments were performed with both HSV-1 and VZV TK. The affinities of the compounds were calculated as K_i values (*Table 3*).

For HSV-1 TK, the guanine derivative **5** and the 2-aminopurine-6-thione derivative **7** show sub-mM range affinity (*Table 3*). The 6-thiopurine analog **6**, which is not phosphorylated by HSV-1 TK, exhibits substantial affinity.

Towards VZV TK, the 2-aminopurine-6(1*H*)-thione (**7**), which is not phosphorylated by the enzyme, binds with an affinity at 0.3 mM. This is comparable to the affinity of ACV (0.2 mM) to HSV-1 TK [26] and represents an inhibitor of VZV TK. The

Table 3. *Binding Affinity Expressed as Inhibition Constants* (K_i) *of Compounds* **1**–**8** *in the Presence of [^{3}H] Thymidine with HSV-1 TK and VZV TK*

Compound	HSV-1 TK K_i/mM	VZV TK K_i/mM
1	5.3 ± 0.28	3.8 ± 0.69
2	2.1 ± 0.63	2.7 ± 0.86
3	4.8 ± 0.28	1.5 ± 0.39
4	3.7 ± 0.20	2.6 ± 0.20
5	0.9 ± 0.07	3.1 ± 0.38
6	1.3 ± 0.20	5.6 ± 1.20
7	0.8 ± 0.34	0.3 ± 0.11
8	2.4 ± 0.25	1.1 ± 0.42

6-(Methylsulfanyl)purine derivative (**8**), which shows a different phosphorylation pattern when presented to HSV-1 TK or VZV TK (*Fig. 2*), exhibits similar binding affinities towards both enzymes. The fact that compound **8** acts as a good substrate, while **6** is only a poor one, suggests that the methylation of the thione at C(6) of the purine ring modifies the polarizability of the base, influencing the catalytic turnover of the VZV TK towards the thione derivatives [30][31].

The 2-amino-6-chloropurine derivative **3** exhibits affinity for VZV TK although it is not phosphorylated (*Fig. 1, b*). The comparison of the compound pairs **2** and **3** as well as **6** and **7** reveals that the NH_2 group at C(2) of the purine ring decreases the phosphorylation but increases the affinities for VZV TK. This effect was not observed for HSV-1 TK. The strongest difference in affinity for the two TKs is found for compound **3**, which has a three-fold higher affinity to VZV than HSV-1 TK, and with **6**, showing a four-fold lower K_i value for HSV-1 TK than VZV TK. These findings provide some insights in structural differences between HSV-1 and VZV TK (the structure of which has not been solved), namely that the structural environment around C(2) of the purine scaffold seems to be much more confined in VZV TK.

A similar comparison of compounds **4** and **5** for both enzymes shows that the presence of an NH_2 group (in **5**) decreases its phosphorylation by VZV TK, without any significant effect on its binding affinity (*Fig. 1* and *Table 3*). In the case of HSV-1 TK, the affinity increases while phosphorylation remains high. Compound **4**, which showed the highest ADP/ATP ratio in HPLC assay (*Fig. 1*), exhibits rather poor binding affinities for both enzymes.

These results do not show a direct correlation between binding affinity (measured as K_i) and catalytic turnover (measured with HPLC). This is in line with the crystallographic results obtained for HSV-1 TK, showing that inhibitor and substrate can share the same binding mode [32], and the recent discovery that k_{cat} depends on the dipole moment of the ligand within the active site [31].

Antiviral tests of compounds **1**–**8** were performed and showed that none of the presented compounds display antiviral activity against *Herpes simplex* virus type 1 and *Varicella-Zoster* virus. This is in agreement with the low affinities measured for all compounds and the data on resistance, showing that antiviral activity is lost when the binding affinity of the prodrug reaches the mM range [33].

Molecular Docking of Compounds **1**–**8** *into the Active Site of HSV-1 TK*. To correlate the structures of compounds and their experimentally determined activities, compounds **1**–**8** were docked into the known active site of HSV-1 TK complexed with **1** [17] (pdb entry 1e2i). The docking program used was AutoDock 3.0 [34], implemented with Lamarckian genetic algorithm. This algorithm docks ligands flexibly and finds independently at every run of docking the lowest-energy conformation of the ligand. Every docking was run ten times and every run was the combination of 1,500,000 energy evaluations. The docking protocol and evaluations were previously described and resulted in the reproduction of the crystallographically observed binding mode [35].

Docking results revealed that none of the eight compounds with sub-mM binding affinities docked into the single binding mode. In comparison, the pyrimidine derivatives that bind in the low-μM range were docked in single and unique binding orientation [35]. The occurrence of multiple-binding modes of the presented purine derivatives **1**–**8** is also reflected by affinities measured by kinetic assays (in the mM–sub-mM range) and is in agreement with the thermodynamic assumption that the higher the binding affinity is, the more definite and unique is the binding mode of the compound. Furthermore, multiple-binding mode was also observed in the X-ray structure of the 9-(2-hydroxypropyl)adenine (**1**) in complex with HSV-1 TK [17].

The criterion to define a compound as a substrate was the number of the docked orientations favorable to phosphorylation from the total number of ten runs. This orientation, named here as 'phosphorylating orientation', is defined as follows: the OH group of the compound (mimicking the 5′-OH group of thymidine (dT)) is pointed towards the γ-phosphate of ATP and is situated at the H-bond distance from the glutamate residue E83. E83 is activating the 5′-OH group of dT [36][26]. The criteria chosen fit the crystallographic evidence shown in the crystal structure of HSV-1 TK complexed with ACV (pdb entry 2ki5) [18], and with compound **1** (pdb entry 1e2i) [17]. The structure of HSV-1 TK with ACV reveals that the side chain of ACV can assume two distinct orientations. In one conformation, the 5′-OH is pointing towards E83 (phosphorylating orientation), in the second conformation towards E225 and Y101, and does not allow phosphorylation.

The crystal structure of 9-(2-Hydroxypropyl)adenine (**1**) in complex with HSV-1 TK revealed two distinct binding modes for the compound [17]; however, the 5′-OH mimicking moiety is always in the phosphorylating position. The position of 5′-OH with respect to E83 is a necessary but not a sufficient condition for a ligand to be a substrate, as shown in previously published data [18][32]. In this procedure, we suggest that a low number (1 to 2) of phosphorylating orientations corresponds to a low probability of the compound being a substrate. A high number (4 and more) suggests the characteristics of the substrate.

Docking of compound **1** reflected the crystal structure and showed distinct multiple-binding mode of the compound. *Fig. 3, a*, shows the only representative orientation of docking of **1**, superimposed to its two crystallographically determined orientations. It confirms that compound **1** is a very poor substrate.

The 2-amino-6-chloropurine (**3**), purine-6(1*H*)-thione (**6**), and thio derivative **7** assume only 1 to 2 orientations, in which the 5′-mimicking OH points towards E83 and γ-phosphate of ATP, while compounds **2** and **5** docked 3 times out of 10 runs into the

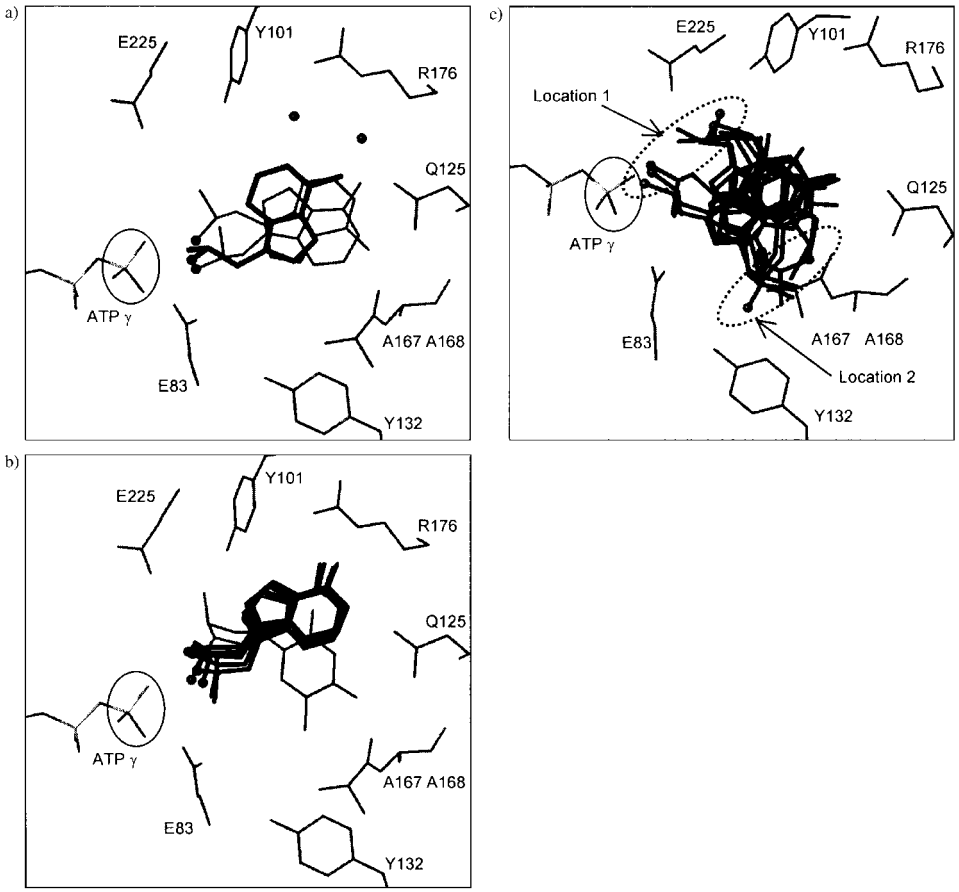

Fig. 3. *Representative docking of 9-(2-hydroxypropyl)-substituted purine derivatives.* All docking poses are represented in thick sticks and their 5′-mimicking OH group in form of balls. γ-Phosphate of ATP is encircled. Amino acid residues forming the binding site are labeled and displayed as thin sticks. For sake of clarity, M128 and Y172, forming the sandwich-like complex with the base, as well as other close residues, have been omitted. Two crystal H_2O molecules, when kept in the active site during docking, are represented as discrete balls. *a*) Docking orientation of 9-(2-Hydroxypropyl)adenine (**1**) favorable for phosphorylation, superimposed to its crystal structure in HSV-1 TK (thin line). *b*) Five lowest-energy conformations of 9-(2-hydroxypropyl)hypoxanthine (**4**) and the thymidine orientation in the crystal structure (thin line). *c*) Docking orientations of 9-(2-hydroxypropyl)purine-6-(methylsulfanyl)purine (**8**). *Location 1:* 5′-mimicking OH groups point towards E225 (whence MeS groups towards A167 and Y132). *Location 2:* 5′-mimicking OH groups point towards A167 and Y132 (whence MeS groups towards E225).

phosphorylating orientation. Finally, the 9-(2-hydroxypropyl)hypoxanthine **4** docked with 5 phosphorylating orientations out of 10 (*Fig. 3, b*). The analysis of the docking orientations in terms of phosphorylation clearly indicates that the substrate characteristics of the compounds, ranging from poor to moderate, are in agreement with the experimental results.

Derivatives **4** and **5** docked in the way that the OH groups point towards the phosphorylation region and the O-atoms at C(6) towards Q125 and R176 (see *Fig. 3,b*,

for **4**), which is very similar to the positioning of the same atoms of thymidine in its crystal structure in HSV-1 TK. This highlights once again the challenge of these compounds to be new HSV-1 TK substrates, *de facto* prodrugs, and supports the results from the phosphorylation assays using the HPLC. Finally, docking of compound **8** revealed it as a poor substrate to HSV-1 TK. In 3 out of 10 runs, the OH group is oriented towards the γ-phosphate but not towards E83. This is in agreement with the fact that (methylsulfanyl)purine derivative **8**, together with thiopurine derivatives **6** and **7**, act as inhibitors of HSV-1 TK. Like the C=S group of compound **6** and **7**, the MeS group in compound **8** is found in two possible locations (see *Locations 1* and *2* in *Fig. 3, c*). One of these locations (*Location 2*), near alanins 167 and 168, is the hydrophobic pocket of HSV-1 TK, in which the Me group as well as bulkier substituents at C(5) of uridine base are positioned, *e.g.*, the bromovinyl group of (bromovinyl)-deoxyuridine (BVDU) [18]. According to the results of kinetic and phosphorylation assays, this suggests that compound **8** is a new putative VZV TK ligand. In analogy to the strong binding of BVDU to VZV TK, we can postulate a binding mode of **8**, which would allow orientation of the 5′-OH mimicking group towards the γ-phosphate and E83, and would be slightly different compared to the one found for HSV1 TK.

Conclusions. – The aim of the presented work was to broaden the knowledge on substrate acceptance of HSV-1 TK and VZV TK, using a series of novel 9-(2-hydroxypropyl)purine derivatives. Indeed, the results show that a variety of purine analogs can bind to the enzymes studied. A set of criteria for the evaluation of molecular docking results – based on the number of phosphorylating orientations – was developed and allows a qualitative prediction of a given ligand to be a good or poor substrate or a potential inhibitor. In particular, docking results showing multiple binding modes for the investigated substances are in line with the observations that these ligands bind to thymidine kinases at mM or sub-mM affinities and show a non-uniform phosphorylation pattern. Furthermore, information derived from multiple binding mode allows the mapping of the active site with respect to chemical space. Thus, it permits the generation of a new pharmacophore hypothesis for the development of better ligands [37].

With regard to suicide gene therapy in the context of stem cell transplantation (SCT) (*e.g.*, bone marrow transplantation), it would be advantageous to have at one's disposal additional compounds that are not used in antiviral therapy. Given the fact that a tailor-made enzyme can be engineered for a specific compound [38][39], the presented weak substrates of thymidine kinases may represent new potential leads in the improvement of the suicide gene therapy in SCT.

Experimental Part

General. [1′,2′-$^3H_2$]Thymidine (3TBq/mmol) was obtained from *Amershan Life Science*. Reagents for enzyme assays were obtained from *Sigma*. M.p.: *Fisher-Jones* instrument; uncorrected. TLC: precoated *Merck* silica gel *60F-254* plates; the spots detected under UV light (254 nm). Column chromatography (CC): silica gel (0.05 – 0.2 mm, *Merck*); glass column was slurry-packed under gravity. Solvent systems used for TLC and CC: S_1: CH_2Cl_2/MeOH 5 : 1; S_2: CH_2Cl_2/MeOH 4 : 1; S_3: CH_2Cl_2/MeOH 3 : 1; S_4: CH_2Cl_2/MeOH/conc. NH_4OH 6 : 6 : 0.3. UV/VIS Spectra: *Hewlett-Packard 8452* spectrometer, 190 – 820 nm range. ^{1}H- and ^{13}C-NMR Spectra: *Varian Gemini-300* spectrometer, operating at 75.46 MHz for the ^{13}C resonance; the samples were dissolved in

(D_6)DMSO and measured at 21° in 5-mm NMR tubes; the ^1H and ^{13}C chemical-shift values are in ppm, referred to TMS. MS: electron impact (EI) mass spectra with *Varian MAT-95* with ionizing energy 70 eV.

9-(2-Hydroxypropyl)adenine (**1**). Preparation of enantiomers of **1** has been reported in [23][40][41].

6-Chloro-9-(2-hydroxypropyl)purine (**2**). A soln. of 6-chloropurine (2.63 g, 17.0 mmol), propylene carbonate (2.16 ml, 25.5 mol), and pulverized NaOH (0.04 g) in anh. DMF (34 ml) was heated under reflux with stirring for 10 h. After filtration, the soln. was concentrated *in vacuo*, and the residual DMF and propylene carbonate were removed by distillation (0.01 Torr) at 70°. Crude product (2.15 g) was purified by CC (S_1) to provide **2** (197 mg, 5%) M.p. 96–97°. UV: 266 (3.98), 208 (4.27). MS: 212 (M^+). Anal. calc. for $C_8H_9ClN_4O$: C 45.27, H 4.28, N 26.42; found: C 45.23, H 4.30, N 26.38. Both enantiomers of **2** have been prepared by synthon approach with (−)-D- and (+)-L-lactic acid as starting material [41].

2-Amino-6-chloro-9-(2-hydroxypropyl)purine (**3**). A soln. of 2-amino-6-chloropurine (1.53 g, 9.0 mmol), propylene carbonate (1.53 ml, 18.0 mol), and pulverized NaOH (0.03 g) in anh. DMF (27 ml) was heated under reflux with stirring for 4.5 h. The mixture was treated in the same way as described for **2**. The residue was submitted to CC (S_2) to yield **3** (847 mg, 41%). Recrystallization of the separated product (360 mg) from i-PrOH afforded colorless crystals of **3** (332 mg, 92%). M.p. 99–101°. UV: 310 (3.77), 248 (3.62), 224 (4.35). MS: 227 (M^+). Anal. calc. for $C_8H_{10}ClN_5O$: C 42.28, H 4.44, N 30.84; found: C 42.23, H 4.43, N 30.87.

9-(2-Hydroxypropyl)hypoxanthine (**4**). A soln. of **2** (532 mg, 2.5 mmol) in 2.5M HCl (7.5 ml, 18.75 mmol) was heated under reflux for 2 h. The soln. was neutralized with 7.5% aq. $NaHCO_3$ (24.5 ml) and evaporated. The crude product was submitted to CC (S_4) to give pure **4** (210 mg, 43%). M.p. 205–207°. Recrystallization from EtOH/H_2O 10:1 afforded anal. samples of **4**. UV: 250 (4.68), 206 (4.56). MS: 194 (M^+). Anal. calc. for $C_8H_{10}N_4O_2$: C 49.46, H 5.19, N 28.86; found: C 49.41, H 5.15, N 28.82.

9-(2-Hydroxypropyl)guanine (**5**). A soln. of **3** (455 mg, 2.0 mmol) in 2.5M HCl (6.0 ml, 15.0 mmol) was heated under reflux for 2 h. The soln. was neutralized with 7.5% aq. $NaHCO_3$ (15.5 ml) and cooled with ice. The solid was filtered off and washed well with cold H_2O to give pure **5** (274 mg, 66%). Recrystallization from H_2O/EtOH 3:1 gave crystals of **5** (87 mg, 32%). M.p. >300°. UV: 254 (4.09), 208 (4.13). MS: 209 (M^+). Anal. calc. for $C_8H_{11}N_5O_2$: C 45.91, H 5.30, N 33.49; found: C 45.84, H 5.27, N 33.53.

9-(2-Hydroxypropyl)purine-6(1H)-thione (**6**). A soln. of **2** (1 g, 4.70 mmol), thiourea (1.39 g, 18.3 mmol), and 4 drops of HCOOH in anh. EtOH (45 ml) was heated under reflux for 1 h. After cooling the mixture, the crystals obtained were filtered off. The crude product was recrystallized twice from H_2O/EtOH 10:1 to give **6** (290 mg, 29%). M.p. 298–300°. MS: 210 (M^+). Anal. calc. for $C_8H_{10}N_4OS$: C 45.70, H 4.80, N 26.65; found: C 45.63, H 4.82, N 26.68.

2-Amino-9-(2-hydroxypropyl)purine-6(1H)-thione (**7**). To a soln. of **3** (820 mg, 3.6 mmol) in anh. EtOH (36 ml) was added thiourea (1.08 g, 14.2 mmol), followed by 5 drops of HCOOH. The soln. was heated under reflux for 1 h and then concentrated *in vacuo*. The crude product was purified by CC (S_3) to yield pure **6** (518 mg, 64%). Recrystallization from EtOH/H_2O 3:1 afforded colorless crystals of **6** (165 mg, 32%). M.p. 284–286°. UV: 346 (4.41), 268 (3.80), 230 (4.09), 210 (4.37). MS: 225 (M^+). Anal. calc. for $C_8H_{11}N_5OS$: C 42.65, H 4.93, N 31.11; found: C 42.60, H 4.96, N 31.13.

9-(2-Hydroxypropyl)purine-6-(methylsulfanyl)purine (**8**). A soln. of **6** (184 mg, 0.88 mmol), NaOH (35 mg, 0.88 mmol), and Me_2SO_4 (111 mg, 0.88 mmol) in H_2O (5 ml) was stirred at r.t. for 1.5 h. The soln. was then neutralized with NaOH soln. and evaporated. The residue was purified by CC (S_1) to give crystals of **8** (117 mg, 60%). M.p. 142–143°. Recrystallization from EtOH/petroleum ether 10:1 afforded anal. samples of **8**. MS: 224 (M^+). Anal. calc. for $C_9H_{12}N_4OS$: C 48.19, H 5.40, N 24.99 found: C 48.23, H 5.39, N 24.96.

Expression and Purification of the HSV-1 and VZV TKs. Competent *E. coli* BL21 were used to express both enzymes. HSV-1 TK and VZV TK were purified according to the protocol described by *Fetzer et al.* [42] and *Amrhein et al.* [27], respectively. Purification was monitored by SDS-PAGE and led to >90% pure HSV-1 TK, which was directly used for kinetic studies. For VZV TK, the *PreScission*® protease cleavage protocol (*Pharmacia*) was applied and led to >95% pure protein. Total protein concentration was measured using the *Bio-Rad Protein Assay* (*Bio-Rad*).

HPLC Assay for Monitoring the Phosphorylation of Substrates **1**–**7**. HPLC was applied to monitor ADP formation during phosphorylation, as previously published [26]. Reactions were carried out in a final volume of 75 µl containing 50 mM *Tris* (pH 7.2), 5 mM ATP, 5 mM $MgCl_2$, 6 mM compound, and 4 µg HSV-1 or VZV TK. The reaction was stopped after 1 h at 37° by a ten-fold dilution in H_2O. The injection volume used for HPLC was 20 µl. The monophosphorylation was monitored qualitatively by the formation of new peaks corresponding to ADP and the monophosphate of the compound. Two different blank reactions (no enzyme or no compound) were performed under the same experimental conditions. The relative ADP formation amount during phosphorylation reaction was monitored. The ratio ADP/ATP was used for the quant. evaluation of the results.

The area under the peak of ATP served as normalization factor. The system allowed the proper separation of the substances by eluting ADP after 5 min and ATP after 10 min. All compounds were separated base-line to base-line, except for compound **4**, which eluted base-line to valley. The detection limit for phosphorylated product lies under 20 nmol [26].

Spectrophotometric Assay for Compound **8**. A UV test was employed to monitor ADP formation during substrate phosphorylation. Enzyme activity was measured with a lactate dehydrogenase/pyruvate-kinase-coupled assay [29]. The change in A_{340} was measured over time at 37°. Plot of the ADP formation velocity in the presence of either 4 μg HSV-1 TK or 4 μg VZV TK is depicted at 6 mM concentration of the substrate. The presented values are mean values of 3 independent measurements and take into account the spontaneous hydrolysis of ATP to ADP.

Binding-Affinity Determination. Inhibition kinetics measuring the conversion of labeled thymidine to thymidine monophosphate in the presence of various concentrations of the respective compound **1**–**8** were performed using the DEAE-cellulose method, as described in [43]. Reactions were carried out in a final volume of 30 μl containing 50 mM *Tris* (pH 7.2), 5 mM ATP, 5 mM $MgCl_2$, and 1.5 mg/ml BSA. The amount of enzyme and concentrations of [^{3}H]thymidine was chosen in consideration of *Michaelis–Menten* conditions for initial velocity measurements. The concentration of the compound was varied according to their affinity towards thymidine kinase. The K_i values were determined by a nonlinear fit of the raw data to *Eqn. 1*

$$V = \frac{V_{max} \cdot [dT]}{K_m \cdot \left(1 + \frac{[\text{compd.}]}{K_1 + [dT]}\right)} \quad (1)$$

for competitive inhibition using the Systat 5.02 software (*Systat Inc.*, Evanston, IL, USA). These values were measured based on at least two independent assays.

Docking into the Active Site of HSV-1 TK. Automated docking simulations were performed with AutoDock 3.0 (*Scripps Research Institute*, La Jolla, CA) by using the HSV-1 TK-thymidine crystal structure (pdb entry 1e2i) [17] to generate atomic energy grids. In this docking site, ATP was modeled starting from ADP of the structure of dTMP-ADP-HSV-1 TK complex (pdb entry 1vtk) [44]. Atomic partial charges were assigned to the protein atoms by means of the AMBER [45] united atom-charge set with SYBYL 6.7 (*Tripos, Inc.*, St. Louis, MO). AMBER-Consistent atomic partial charges assigned to compounds **1**–**8** were calculated *ab-initio* according to the protocol in [45]. Atomic interaction energy grids were calculated with the AutoGrid 3.0 routing of AutoDock 3.0 and probes corresponding to each atomic type found in the ligand, at 0.35-Å grid positions in a 60-Å cubic box centered on the HSV-1 TK active site. Docking was performed by previously evaluated protocols [35]. Calculations were performed on a *SGI* O_2 graphic workstation.

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