Research Article

N^2-Phenyl-9-(hydroxyalkyl)guanines and related compounds are substrates for Herpes simplex virus thymidine kinases

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Abstract

Herpes simplex virus (HSV) types 1 and 2 thymidine kinases (TK) are responsible for phosphorylation of antiherpes acyclonucleosides such as acyclovir (ACV) and 9-(4-hydroxybutyl)guanine (HBG). Related compounds, the N^2-phenyl-9-(hydroxyalkyl)guanines, are devoid of direct antiviral activity, but potently inhibit the viral TKs and block viral reactivation from latency *in vivo*. The similarity in structure between the acyclonucleosides and TK inhibitors raised the question of the relevance of phosphorylation of certain of the latter analogs in their mechanisms of action. Using recombinant TKs and HPLC analysis of reaction mixtures, we report that the lead TK inhibitor N^2-phenyl-9-(4-hydroxybutyl)guanine (HBPG) and its pentyl homolog (HPnPG) are excellent substrates for the enzymes, approaching the efficiency with which the natural substrate thymidine is phosphorylated, and significantly better than ACV or HBG. Other 9-hydroxyalkyl congeners are substrates for the enzymes, but with much poorer efficiency. HBPG triphosphate was a poor inhibitor of HSV DNA polymerase, the target of acyclonucleoside triphosphates, suggesting that phosphorylation of HBPG is not important in its mechanism of blocking viral reactivation *in vivo*. The fact that HBPG is an efficient substrate is consistent, however, with its binding mode based both on molecular modeling studies and x-ray structure of the HBPG:TK complex.

Introduction

We recently reported that a potent inhibitor of Herpes simplex virus (HSV) types 1 and 2 thymidine kinases (TK), N^2-phenyl-9-(4-hydroxybutyl)guanine (HBPG; see structures), is an efficient substrate for the enzymes (Manikowski *et al.* 2005). In addition, this compound was the only derivative among a related series that demonstrated potent antiviral activity against experimental models of HSV reactivation and encephalitis in mice (Manikowski *et al.* 2005). In order to evaluate the significance of phosphorylation of HBPG in its mechanism of activity *in vivo*, we have compared the kinetic parameters of phosphorylation of HBPG with those for the natural substrate thymidine (TdR) and the antiherpes drug acyclovir (ACV). In addition, we screened related compounds for their ability to act as substrates for the HSV TKs. Finally, we compared the inhibitory potencies of HBPG-MP and the synthetic triphosphate HBPG-TP with that of ACV-TP on HSV-1 DNA polymerase. The results are consistent with the probability that HBPG does not owe its anti-reactivation activity in the animal models to its conversion to the monophosphate form in virus-infected cells.

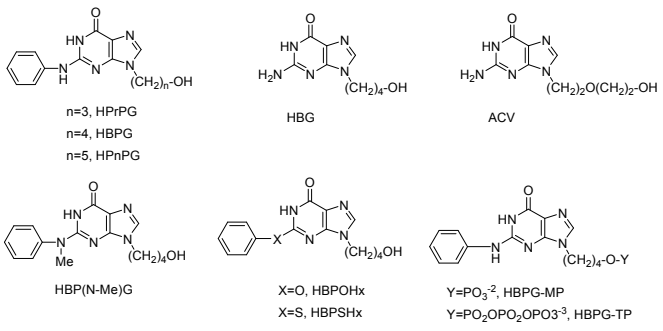

Figure 1. Structures and acronyms of TK inhibitors.

Material and Methods

Chemical compounds

Synthesis of N^2 phenyl-9-(hydroxyalkyl)-6-oxopurines and HBPG-MP are described in Manikowski *et al.* (2005). 9-(4-Hydroxybutyl) guanine (HBG) was prepared as described by Larsson *et al.* (1983). Acyclovir mono and triphosphates (ACV-MP and ACV-TP) were prepared by the method of Sawai et al. (2002); the products were identical with those reported by Furman *et al.* (1979).

N^2-Phenyl-9-(4-triphosphoryloxybutyl)guanine sodium salt, HBPG-TP, was prepared as follows. The free acid of HBPG-MP (47 mg, 123 mmol) was converted to its trioctylammonium salt by dissolving it in N,N-dimethylformamide (5 mL) containing trioctylamine (1.5 eq., 65 mg, 184 mmol), followed by evaporation of the solvent to dryness. A suspension of the residue in hexamethylphosphoric triamide (HMPA) (680 mL) was treated with 1,1'-carbonyldiimidazole (100 mg, 613 mmol) under N_2, and, after 3.5 h, the clear reaction mixture was quenched by the addition of MeOH (92 mL). A solution of trioctylammonium pyrophosphate (613 mmol) [prepared from tetrasodium pyrophosphate (163 mg, 613 mmol) and trioctylamine (542 mg, 1.53 mmol)] in HMPA (4.9 mL)] was added dropwise with stirring. Precipitation occurred almost immediately. After 24 h at rt, the mixture was quenched with 0.02 M aqueous triethylammonium bicarbonate (15 mL) and extracted with Et_2O (2 x 20 mL). The aqueous material was applied to a DEAE-Sephadex column (20 x 2.2 cm), bicarbonate form, and elution was carried out in a linear gradient of 0.05-1.0 M aqueous triethylammonium bicarbonate, pH 7.9, at a flow rate of 4.0 mL/min, with detection at 260 nm. The product eluted in fractions 86-97 (15 mL/fraction). The fractions were combined and coevaporated with 1-butanol at 30 ^oC under vacuum, and the resulting material was dissolved in water and passed through 15 mL of Dowex 50Wx8, Na^+ form. The eluate was lyophilized, the residue dissolved in water, and passed over chelating resin (Chelex 100). The eluate was lyophilized to give 27 mg (36%) of HBPG-TP as the sodium salt. ^1H NMR (300 MHz, D_2O) d 7.84 (s, 1H), 7.49 (m, 2H), 7.38 (m, 2H), 7.14 (m, 1H), 4.08 (t, 2H, J = 7.0 Hz), 3.97 (q, 2H, J = 6.4 Hz), 1.97-1.86 (m, 2H), 1.67-1.53 (m, 2H); ^{31}P NMR (121 MHz, D_2O) d -4.82 (d, J = 19.3 Hz), -9.37 (d, J = 19.3 Hz), -20.76 (t, J = 19.3 Hz).

HSV thymidine kinases and substrate assay

HSV-1 and HSV-2 TKs were expressed as His_6-tagged proteins and assayed as described (Manikowski *et al.* 2005). One unit (U) is the amount of enzyme which phosphorylates 1 nmol of TdR to TMP in 1 hour at 37 °C. Test compounds (200 μM) were incubated at 37 °C for various times in 25 μL of a mixture containing 30 mM HEPES-K^+, pH 7.5, 6 mM $MgCl_2$, 6 mM ATP, 0.5 mM DTT and approximately 2 U of HSV-1 TK or 10 U of HSV-2 TK. Samples were heated at 100 °C for 5 min and centrifuged for 15 min at 10,000 rpm in a microcentrifuge. Supernatants were transferred to a new tube for subsequent HPLC analysis. Reverse phase chromatography employing a Shimadzu HPLC system was used to separate test compounds from phosphorylated products. A 4.6 mm x 25 cm ALL-TIMA C18-NUC 100A 5U (Alltech) column was used at rt in the following conditions: injection volume, 20 μL; detection, UV 260 nm; eluents, buffer A (20 mM KH_2PO_4, pH 7.5), buffer B (20 mM KH_2PO_4, pH 5.2, 60% MeOH); linear gradient, 30 min from 0% to 100% buffer B; 20 min 100% buffer B; flow rate, 0.5 ml/min. Retention times (R_t) for compounds and their monophosphates are summarized in Table 1.

HSV-1 DNA polymerase and assay

HSV-1 DNA polymerase was purified from HSV-1 infected HeLa cells and assayed as previously described (Focher *et al.* 1992). Inhibitor studies (K_i determinations) were done using the above assay conditions with [^3H]dGTP (1,500 cpm/pmol) at five concentrations (between 1.5 and 10 μM), in the presence of increasing concentrations of inhibitor.

Results

Substrate assays of HSV thymidine kinases

Thymidine kinases were prepared by expressing the cloned genes for HSV-1 and HSV-2 enzymes containing a His_6 tag (Manikowski *et al.* 2005). Properties, including K_m values for the substrate TdR and inhibitor sensitivities, were similar to those observed with enzymes isolated from virus-infected cells (Focher *et al.* 1988, Hildebrand *et al.* 1990).

In the assays of analogs with the enzymes, each analog (200 μM) was incubated for the indicated time in the assay mixture, and the products of the reaction were resolved by HPLC as described in the Experimental Section. Synthetic HBPG-MP was used to confirm the retention time (R_t) of the product, and other monophosphates were identified by their earlier retention times compared with those of the test analogs. Figure 2 illustrates the time-course of phosphorylation of HBPG and the antiherpes compound HBG (Larsson *et al.* 1983) by HSV-1 TK, resulting in nearly complete conversion of the former compound but only ca. 3% conversion of HBG, emphasizing the profound effect of the phenyl group of HBPG on substrate effi-

ciency. Indeed, 10 units of the enzyme were used in these assays to reveal the weak substrate property of HBG.

Table 1 summarizes the chromatographic behavior of analog:monophosphate pairs and the degrees of the conversion of each to its monophosphate catalyzed by HSV-1 and HSV-2 TKs under standard incubation conditions after 40 minutes incubation. 100% corresponds to conversion of 5 nmoles of substrate to monophosphate. These results showed that HBPG and the related homolog HPnPG were the best substrates for both enzymes, while the lower homolog HPrPG appeared not to be phosphorylated under these conditions. The latter result is consistent with the rigid fit between the TK substrate and phosphorylation (ATP) sites in the enzyme (Bennett *et al.* 1999). 9-Hydroxybutyl compounds lacking the 2-NH group, the

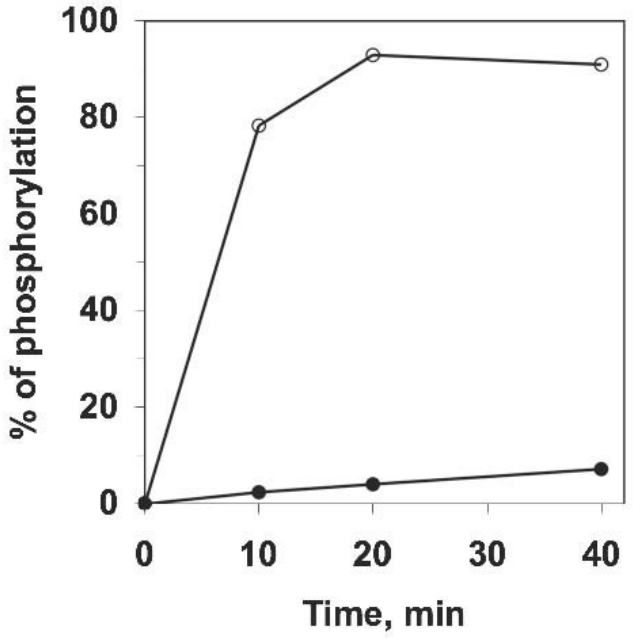

Figure 2. Kinetics of phosphorylation of HBPG (o) and HBG (●) by HSV-1 TK. Reactions were carried out as described in the Experimental Section, using 200 μM of compounds and 10 U of enzyme (in order to measure conversion of HBG). Aliquots of mixtures were analyzed by HPLC, and the % conversion to monophosphate is plotted against time.

hypoxanthines HBPOHx and HBPSHx, are potent inhibitors of the TKs (Manikowski *et al.* 2005) and moderate substrates for the enzymes, while the N^2-methylated analog HBP(N-Me)G, although a weak TK inhibitor (Manikowski *et al.* 2005), also was a moderate substrate for the enzymes (Table 1). These results indicate that it is the position of the ω-hydroxyl group in proximity to the γ-phosphoryl group of ATP in the enzyme:compound complex that enables the compound to be a substrate.

Comparison of kinetic parameters of ACV and HBPG with the substrate TdR

As summarized by the results of Table 2 the HSV TKs efficiently phosphorylate the natural substrate TdR, with V_{max}/K_m of 17 and 7.7 for types 1 and 2 enzymes, respectively. ACV is actually a poor substrate for the enzymes, as evidenced by its very low values of V_{max}/K_m. HBPG, on the other hand, is comparable with the natural substrate in the efficiency with which it is phosphorylated. The poor substrate property of ACV has been reported (Keller *et al.* 1981), and the compound owes its potent and selective antiherpes effect to the efficiency with which the limited ACV-MP formed is further activated to the triphosphate, which is a po-

Table 1. Conversion of 9-hydroxyalkyl-6-oxopurines to monophosphates (MP) by HSV TKs.

Cpd acronym	HPLC R_t, min[1]		Relative % MP[2]	
	Parent	MP	HSV1 TK	HSV2 TK
HBPG	38.46	31.79	100[3]	100[3]
ACV	25.18	14.56	32	5
HBG[4]	20.36	13.25	3	14
HPrPG	38.26	nd	nd	nd
HPnPG	44.22	36.01	124	58
HBP(N-Me)G	39.66	33.34	15	11
HBPOHx	36.23	29.82	22	16
HBPSHx	40.70	34.35	12	5

[1]R_t = retention time in min; HPLC conditions in Experimental Section. [2]Cpds assayed at 200 mM for 40 min in the presence of ca. 2 U of HSV-1 TK or 8 U of HSV-2 TK. [3]100% for HBPG corresponds to 25% conversion to HBPG-MP for both enzymes. [4]Assayed with 10 U of enzymes. nd, not detected.

Table 2. Kinetic parameters for phosphorylation of compounds by HSV TKs.

Enzyme	Cpd[1]	K_m, mM	V_{max}, mmol/h/mL	V_{max}/K_m
HSV1 TK	HBPG	12±3	190±15	16
	ACV	122±27	18±2	0.15
	TdR	1.0±0.2	17±2	17
HSV2 TK	HBPG	9.3±0.5	121±2	13
	ACV	200±17	9.0±0.5	0.045
	TdR	2.0±0.2	15.3±0.5	7.7

[1]Assays for HBPG and ACV were done with the HPLC method; those for TdR were done with [^{3}H]TdR and the filter method (Manikowski et al, 2005).

tent, dead-end substrate inhibitor of the HSV DNA polymerase (Reardon & Spector 1989). Given the efficiency with which HPBG is phosphorylated to its monophosphate by HSV TKs, it was logical to consider the results of a parallel pathway to "activation" of this compound to its triphosphate.

Inhibition of HSV-1 DNA polymerase by nucleotide analogs

In order to evaluate whether HBPG inhibits viral reactivation through the inhibition of DNA synthesis by its phosphorylated derivatives, we synthesized HBPG-MP (Manikowski *et al.* 2005) and HBPG-TP and tested their effects as possible inhibitors of HSV1 DNA polymerase *in vitro*. Each compound was tested for inhibition of viral polymerase activity in the presence of 5 μM [^{3}H]dGTP (K_m 0.7 μM). Under these conditions HBPG-MP and HBPG-TP were weak DNA polymerase inhibitors, with IC_{50} values of 0.07 mM and 1.1 mM, respectively. Under the same conditions, ACV-TP potently inhibited the enzyme with IC_{50} of 7 μM. ACV-TP has been reported to be competitive with dGTP as an inhibitor of HSV DNA polymerase (Reardon & Spector 1989). However, when assayed at variable concentrations of dGTP, HBPG-MP and HBPG-TP inhibited this enzyme in a non-competitive manner, resulting in K_i values of 90 and 800 μM, respectively (data not shown). Thus, not only are the HBPG phosphates weak inhibitors of HSV-1 DNA polymerase, but they also appear to act by a mechanism inconsistent with that of antiviral triphosphates.

Discussion

In this work we report that the HSV TK inhibitor HBPG and closely related compounds are efficient substrates for the types 1 and 2 enzymes. HBPG is the prototype of a series of specific TK inhibitors including many which cannot act as substrates for the enzymes, e.g. N^2-phenylguanines lacking a 9-substituent such as N^2-(3-[trifluoromethyl]phenyl)guanine (cpd. 122E). It has been thought that the ability of these compounds to block HSV reactivation did not depend on the substrate property of the compounds. Indeed, the most potent guanine derivative 122E also blocked HSV reactivation in a mouse model (Yanachkov *et al.* 2011).

Compounds that are HSV TK substrates and have direct antiHSV activity include analogs of thymidine and "acyclonucleosides" such as acyclovir (ACV) and its carba-isostere HBG. ACV (Keller *et al.* 1981) and HBG (Figure 1) are indeed poor substrates for the HSV TKs (see also Table 2), but the low levels of monophosphates formed in HSV-infected cells can readily be converted to di- and tri-phosphates, the latter being the ultimate, potent active antiviral forms (Furman *et al.* 1979, Larsson *et al.* 1983). Considering that the triphosphate form of HBPG is not a potent HSV DNA polymerase inhibitor (see above) and that non-substrate inhibitors possess anti-reactivation activity *in vivo* (Yanachkov *et al.* 2011), we conclude that phosphorylation of HBPG is not required for its anti-reactivation and, possibly, anti-encephalitic activity *in vivo*.

The results reported here, however, are fully consistent with the similar substrate-competent binding modes of HBPG and ACV (and Thd) observed in the solid state structures of HSV-1 TK (Bennett *et al.* 1999). It is also consistent with the results of molecular modeling studies (Focher *et al.* 2011) in which 9-substituted N^2-phenylguanines were predicted to bind HSV TKs in the substrate mode.

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