Kinetic studies with *N*²-phenylguanines and with L-thymidine indicate that Herpes simplex virus type-1 thymidine kinase and thymidylate kinase share a common active site

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It is known that the Herpes simplex virus type 1 (HSV-1)-encoded thymidine kinase (TK) co-purifies with an associated thymidylate kinase (TMPK) activity and that thymidylate (TMP) inhibits the phosphorylation of thymidine by the HSV-1 TK. Here we demonstrate that: (i) TMP phosphorylation catalysed by the viral TMPK is competitively inhibited by thymidine (TdR) with a K_i equal to its K_m as substrate for the viral TK; (ii) L-thymidine (L-TdR), the enantiomer of the naturally occurring D-TdR and a substrate for the HSV-1 TK [Spadari, Maga, Focher, Ciarrocchi, Manservigi, Arcamone, Capobianco, Caruso, Colonna, Iotti and Garbesi (1992) *J. Med. Chem.* **35**, 4214–4220], is a powerful inhibitor of the HSV-1 TMPK activity with a K_i value identical with its K_m as a substrate for the viral

TK; (iii) both viral TK and TMPK activities are inhibited, in a competitive way and with identical K_i values, by novel, non-substrate inhibitors of HSV-1 TK, *N*²-phenylguanines; (iv) L-TdR is phosphorylated to L-TMP by the viral TK, but L-TMP is not phosphorylated to L-TDP by the viral TMPK activity; and (v) L-TMP inhibits competitively and with identical potencies the phosphorylation of TdR and TMP catalysed respectively by the HSV-1 TK and TMPK activities. In conclusion, our data demonstrate that both TK and TMPK activities encoded by HSV-1 share a common active site which is very tolerant in accepting modified nucleosides, but cannot readily accommodate modified nucleoside monophosphates.

INTRODUCTION

Thymidine kinase (TK, EC 2.7.1.21), a ubiquitous enzyme of the salvage pathway of nucleoside metabolism, catalyses the phosphorylation of thymidine (TdR) to thymidylate (TMP) using ATP as the phosphate donor.

In contrast with the cellular TK, the Herpes simplex virus type 1 (HSV-1) TK possesses not only thymidine kinase activity but also deoxycytidine kinase activity, which phosphorylates deoxycytidine to dCMP (Jamieson et al., 1974; Thouless and Wildy, 1975), and thymidylate kinase (TMPK) activity, which phosphorylates TMP to TDP (Cheng, 1976; Cheng et al., 1979). Furthermore, the viral TK, because of its low substrate specificity, can also phosphorylate mono- and/or di-phosphate forms a wide variety of pyrimidine and purine nucleoside analogues (Fyfe et al., 1978; Fyfe, 1982; Cheng and Prusoff, 1986; De Clercq, 1988), which represent most of the anti-herpetic drugs clinically used or under scientific investigation (De Clercq, 1988; Mertz, 1990). These modified nucleoside analogues exert their anti-herpetic activity when converted ultimately into the active triphosphate form by cellular enzymes. In this form they preferentially inhibit the viral DNA polymerase or are incorporated into the viral DNA interfering with the replication or transcription of the viral genome (Wright and Brown, 1990; Balzarini et al., 1990). It is known that the phosphorylation of these analogues from diphosphates to triphosphates is carried out by non-specific cellular nucleoside diphosphate kinases and is not a limiting step in the activation of the drugs. In contrast, the phosphorylation of the nucleosides to monophosphates by the HSV-1 TK and of the mono- to di-phosphates by the HSV-1

TMPK are crucial steps for their activation, since the cellular nucleoside kinases and monophosphate kinases are generally more selective than the viral enzyme in the recognition of the substrate (Cheng and Prusoff, 1986; De Clercq and Walker, 1988; Dorsky and Crumpacker, 1987; Descamps and De Clercq, 1981). Thus, (*E*)-5-(2-bromovinyl)-2'-deoxyuridine (BVdU), which is phosphorylated to BVdUMP by both HSV-1 and HSV-2 TKs, is exceptionally active against HSV-1 proliferation, but displays a modest activity against HSV-2. This is ascribed to the fact that HSV-2 TK, in contrast with HSV-1 TK, lacks a TMPK activity and is thus unable to phosphorylate BVdUMP to BVdUDP. However, not all the nucleoside analogues which are phosphorylated by the HSV-1 TK are by definition substrates for the HSV-1 TMPK. In fact, acyclovir (ACV), which is phosphorylated by the HSV-1 TK to ACV monophosphate, is not further transformed to ACV diphosphate by HSV-1 TMPK but by cellular kinases (Miller and Miller, 1980).

These data suggest that TMPK associated with the HSV-1 TK activity is more stringent than the latter in the acceptance of the substrate, and this raises the question as to whether one or more sites are involved in the phosphorylation of both TdR and TMP catalysed by the HSV-1 TK and TMPK activities.

Since HSV-1-encoded TK and TMPK activities could not be separated by affinity column chromatography, by glycerol-gradient centrifugation or by isoelectric focusing (Cheng and Prusoff, 1978), both activities may reside on the same polypeptide. Kinetic studies by Cheng and co-workers (1979) led to suggestions either that both activities share a common active site, or that two sites exist, but that they overlap. We have reinvestigated this problem by measuring the affinities of some novel non-substrate

Abbreviations used: HSV-1, Herpes simplex type 1; TK, thymidine kinase; TMPK, thymidylate kinase; D- and L-TdR, D- and L-thymidine; BVdU, (*E*)-5-(2-bromovinyl)-2'-deoxyuridine; ACV, acyclovir; DTT, dithiothreitol; PEI, polyethylenimine.

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inhibitors (N^2 -phenylguanines) and substrate inhibitors (L-thymidine) of HSV-1 TK, described by our group (Focher et al., 1988; Spadari and Wright, 1989; Hildebrand et al., 1990; Spadari et al., 1992) for the active site of the HSV-1 TMPK. N^2 -phenylguanines competitively inhibit, in the micromolar range, the phosphorylation of [3 H]thymidine by HSV-1 TK (Focher et al., 1988; Spadari and Wright, 1989; Hildebrand et al., 1990) and also inhibit the reactivation of HSV-1 from infected neuronal ganglia (Leib et al., 1990). On the other hand, L-thymidine not only competitively inhibits the phosphorylation of [3 H]thymidine by HSV-1 TK, but it is phosphorylated to its monophosphate form with a K_m of 2 μ M. Furthermore, at 90 μ M, L-thymidine reduces by more than two orders of magnitude the HSV-1 multiplication.

The studies here described allow to conclude that both TK and TMPK activities encoded by HSV-1 share a common active site.

MATERIALS AND METHODS

Chemicals and enzyme

[3 H]Thymidine ([3 H]TdR; 30 Ci/mmol) was from Amersham International. L-[3 H]Thymidine (L-[3 H]TdR; 6 Ci/mmol) was from Moravsek Biochemicals. [14 C]TMP (40 mCi/mmol) was from ICN Flow. Polyethyleneimine (PEI)-cellulose F plastic sheets (layer thickness 0.1 mm) for t.l.c. were from Merck. All other chemicals and reagents were purchased from local suppliers. TK was purified HeLa TK⁻ infected cells as described by Focher et al. (1988). Membranes and mitochondria still present in the cytoplasm after earlier centrifugations were eliminated by ultracentrifugation at 100000 g for 30 min.

Inhibitors

L-Nucleosides were synthesized as described in Spadari et al. (1992). N^2 -Phenylguanines were synthesized as described by Focher et al. (1988) and Hildebrand et al. (1990).

Thymidine kinase assay

TK was assayed as follows: 0.02 unit of enzyme was incubated at 37 °C for 30 min in 25 μ l of a mixture containing 30 mM potassium Hepes, pH 7.5, 6 mM $MgCl_2$, 6 mM ATP, 0.5 mM dithiothreitol (DTT) and 3.3 μ M [3 H]TdR (1500 c.p.m./pmol) or 10 μ M L-[3 H]TdR (660 c.p.m./pmol). The reaction was terminated by spotting 20 μ l of the incubation mixture on a 25 mm-diameter DEAE-cellulose paper disc (DE-81 paper; Whatman). The disc was washed twice in an excess of 1 mM ammonium formate, pH 3.6, in order to remove unconverted nucleoside and then once in distilled water and finally in ethanol. Radioactive TMP or L-TMP was estimated by scintillation counting in 1 ml of Betamax (ICN-Biomedicals).

One unit of TK is defined as the amount of enzyme which converts, under the above assay conditions, 1 nmol of thymidine into TMP/h.

Thymidylate kinase assay

HSV-1 TK (0.02 unit) was incubated at 37 °C for 60 min in 15 μ l of a mixture containing 30 mM potassium Hepes (pH 7.5), 6 mM $MgCl_2$, 6 mM ATP, 0.5 mM DTT and 50 μ M [14 C]TMP (1000 c.p.m./pmol). Aliquots of 3 μ l were spotted on a PEI t.l.c. sheet. The sheet was developed in 0.5 M ammonium formate (pH 3.6) and the positions of the labelled TMP and TDP were revealed by autoradiography. The sheet was cut and the amount of radioactive TDP formed was estimated by scintillation counting in 1 ml of Betamax.

Figure 1 Double-reciprocal plot of the inhibition of HSV-1 TMPK by TdR as a function of TMP concentration

Assays were done as described in the Materials and methods section in the presence of various concentrations of [14 C]TMP. TdR concentrations: $\circ$, 0; $\blacktriangle$, 1.6 μ M; $\square$, 3.3 μ M; $\bullet$, 8 μ M.

To assay the phosphorylation of [3 H]TdR or L-[3 H]TdR to their diphosphate forms, 0.1 unit of enzyme was incubated at 37 °C for 60 min in 25 μ l of a mixture containing 30 mM potassium Hepes, pH 7.5, 6 mM $MgCl_2$, 6 mM ATP, 0.5 mM DTT and 3.4 μ M [3 H]TdR (1500 c.p.m./pmol) or L-[3 H]TdR (660 c.p.m./pmol). The reaction was terminated by spotting 20 μ l of the incubation mixture on a 25 mm DEAE-cellulose paper disc. The disc was washed twice in an excess of 20 mM ammonium formate, pH 3.6, in order to remove [3 H]TdR and [3 H]TMP, once in distilled water and finally in ethanol. The filter was then dried, and the radioactivity on the filter was estimated by scintillation counting in 1 ml of Betamax. Radioactivity on the filter represents the amount of labelled TDP produced by the action of TK.

RESULTS

TdR is a competitive inhibitor of TMP phosphorylation catalysed by the HSV-1 TMPK

Previous kinetic studies showed that TMP is a product inhibitor of TdR phosphorylation catalysed by the HSV TK, with a K_i identical with its K_m as substrate for the viral TMPK (Cheng et al., 1979). In the reciprocal experiment we have found that TdR is also a competitive inhibitor of the TMP phosphorylation catalysed by HSV-1 TMPK (Figure 1). The K_i calculated from Dixon plot is $1.25 \pm 0.05 \mu$ M, almost identical with its K_m as substrate for the viral TK, which is $1.2 \pm 0.1 \mu$ M.

L-TdR is a competitive inhibitor of HSV-1 TMPK activity and its phosphorylation by the viral TK is inhibited by TMP

We have recently shown that HSV-1 encoded TK, in contrast with the cellular enzyme, is not stereospecific, because it is able to phosphorylate both the naturally occurring D-TdR as well as its enantiomer, L-TdR (Spadari et al., 1992). Thus we have hypothesized that L-TdR, like the natural TdR, should competitively inhibit the viral TMPK with a K_i identical with its K_m as substrate for the viral TK. When competition experiments were performed, we found that L-TdR is a competitive inhibitor of the HSV-1 TMPK (Figure 2), with a K_i calculated from a Dixon plot of $1.0 \pm 0.1 \mu$ M, identical with the K_m value of L-TdR for the HSV-1 TK, which is $1.01 \pm 0.05 \mu$ M. We also found that TMP inhibited L-TdR phosphorylation catalysed by the HSV-1

Figure 2 Double-reciprocal plot of the inhibition of HSV-1 TMPK by L-TdR as a function of TMP concentration

Assays were done as described in the Materials and methods section in the presence of various concentrations of [^{14}C]TMP. L-TdR concentrations: $\circ$, 0; $\blacktriangle$, 1 μM ; $\triangle$, 3 μM ; $\bullet$, 10 μM .

Figure 3 Double-reciprocal plot of the inhibition of HSV-1 TK by TMP as a function of L-TdR concentration

Assays were done as described in the Materials and methods section in the presence of various concentrations of L- ^{3}H]TdpR. TMP concentrations: $\circ$, 0; $\blacktriangle$, 5 μM ; $\square$, 100 μM .

TK (Figure 3) with a K_i of $13 \pm 1 \mu\text{M}$, equal to its K_m as substrate for the viral TMPK, which is $12 \pm 1 \mu\text{M}$.

***N*²-Phenylguanines are specific non-substrate competitive inhibitors of both HSV-1 TK and TMPK activities**

If a single site is involved in the phosphorylation of both TdR and TMP, one would expect that a competitive inhibitor of TdR phosphorylation by the viral TK should act also as a competitive inhibitor of the associated TMPK with an identical K_i value.

To verify this assumption, we challenged the TMPK activity encoded by HSV-1 with a group of *N*²-phenylguanines, synthesized and characterized in our laboratory (Focher et al., 1988; Spadari and Wright, 1989; Hildebrand et al., 1990), which specifically inhibit the viral TK. These compounds are not phosphorylated by the viral TK and, once bound to the active site, they block the enzyme by presumably occupying the TdR-binding site.

These non-substrate inhibitors of HSV-1 TK were found to be competitive inhibitors also of the viral TMPK, as shown in Table 1. The K_i values calculated from Dixon plots are in all cases

Table 1 Kinetic parameters for the competitive inhibition of HSV-1 TK and TMPK activities by some *N*²-phenylguanines.

Abbreviations used: 1m-CF3PG, *m*-trifluoromethylphenylguanine; PhdG, 9-(2'-deoxyribofuranosyl)guanine; p-BrPG, *p*-bromophenylguanine; m-CIPG, *m*-chlorophenylguanine; PhG, phenylguanine; p-OHPG, *p*-hydroxyphenylguanine.

Inhibitor	TK (K_i , μM)	TMPK (K_i , μM)
m-CF3PG	0.2 ± 0.05	0.28 ± 0.05
PhdG	0.33 ± 0.05	0.5 ± 0.05
p-BrPG	1.0 ± 0.2	1.1 ± 0.2
m-CIPG	2.0 ± 0.2	1.5 ± 0.2
PhG	8.0 ± 0.5	7.5 ± 0.5
p-OHPG	140 ± 5	155 ± 5

Figure 4 Kinetics of phosphorylation of [^3H]TdpR and L- ^{3}H]TdpR by HSV-1 TK to their mono- and di-phosphate forms

$\circ$, TDP; $\bullet$, TDP; $\square$, L-TMP; $\blacksquare$, L-TDP.

identical with the ones previously determined for the inhibition of the HSV-1 TK.

L-TMP is not a substrate for HSV-1 TMPK, but it is a competitive inhibitor of both HSV-1 TK and TMPK activities

Since L-TdR is phosphorylated to L-TMP by HSV-1 TK (Spadari et al., 1992) and L-TdR is a competitive inhibitor of the viral TMPK (see above), we investigated the possibility that L-TMP may also be substrate for HSV-1 TMPK. Purified HSV-1 TK/TMPK was thus incubated in presence of 3.4 μM [^3H]TdpR or L- ^{3}H]TdpR as described in the Materials and methods section. Only L-TMP accumulated in the course of the reaction with L-TdR, whereas no detectable amounts of L-TDP were formed during the 60 min of incubation time (Figure 4), even using higher concentrations of enzyme (results not shown). In a control reaction with TdR, both TMP and TDP were formed at appreciable levels (Figure 4). These results raised the possibility that two distinct active sites with different stereospecificities are responsible for TdR and TMP phosphorylation. We have investigated this hypothesis by studying the effect of L-TMP on the phosphorylation of both TdR and TMP catalysed by the HSV-1 TK/TMPK. If two active sites do really exist, L-TMP could act as a non-substrate inhibitor of TdR phosphorylation, as well as a non-substrate inhibitor of TMP phosphorylation, but with different dissociation constants (K_i). Otherwise, L-TMP could be

Table 2 Kinetic parameters for the inhibition of HSV-1 TK and TMPK activities by the D- and L-enantiomers of TdR and TMP

Inhibition was competitive in each case.

Enzyme	Substrate	Inhibitor	K_m (μ M)	K_i (μ M)
TK	TdR	L-TMP	1.20 ± 0.1	680 ± 10
	L-TdR	TMP	1.01 ± 0.05	13 ± 1
TMPK	TMP	TdR	12 ± 1	1.25 ± 0.05
		L-TdR	12 ± 1	1 ± 0.2
		L-TMP	12 ± 1	700 ± 10

a non-competitive inhibitor of TMP phosphorylation, simply acting as a negative allosteric regulator via its interaction with the active site responsible for TdR phosphorylation. In this case the dissociation constant (K_i) for the competitive inhibition of TdR phosphorylation and the non-competitive inhibition of TMP phosphorylation should be equal, because L-TMP interacts with a single active site. On the other hand, if a common active site is involved in the phosphorylation of TdR and TMP, one would expect L-TMP to act as a competitive inhibitor against both substrates, with the same K_i .

The results of our experiments are summarized in Table 2. L-TMP is a competitive inhibitor of the phosphorylation of both TdR and TMP catalysed respectively by the HSV-1 TK and TMPK activities; the K_i values for inhibition of the viral TK and TMPK activities by L-TMP are identical, $680 \pm 10 \mu$ M and $700 \pm 10 \mu$ M respectively, indicating that L-TMP competes with both substrates for a common active site.

DISCUSSION

We have co-purified, through ion-exchange and affinity column chromatography, the viral TK and TMPK activities from HSV-1 infected HeLa TK⁻ cells, as previously described (Focher et al., 1988), and we have investigated whether one or more active sites were involved in the phosphorylation of TdR and TMP by a double approach. First, we have studied the kinetics of interaction of nucleosides, which are substrates for the HSV-1 TK, with the HSV-1 TMPK activity, both of the natural D configuration and of the unnatural L configuration. Secondly, we have tested as inhibitors of the HSV-1 TMPK, a series of highly specific non-substrate competitive inhibitors of the HSV-1 TK, the *N*²-phenylguanines, developed and extensively characterized by us (Focher et al., 1988; Spadari and Wright, 1989; Hildebrand et al., 1990).

All data reported here can be explained in a satisfactory way only assuming that a common active site is involved in the phosphorylation of both TdR and TMP. When we studied the interaction of L-TMP with the HSV-1 TMPK activity, we obtained data showing that L-TdR, after being converted into L-TMP by the viral TK, is not further phosphorylated to L-TDP by the associated TMPK activity. According to the hypothesis that a single active site is shared by both the TK and TMPK

activities, the dissociation constants of L-TMP derived from Dixon plots are identical (680 and 700 μ M respectively). The low affinity of L-TMP for the active site of the viral TK and TMPK activities can explain why we were not able to detect L-TDP in our assays in the presence of 10 μ M L-TdR as substrate. It has been shown (Cheng et al., 1979) that the first phosphorylated product of the reaction, TMP, leaves the active site and that it has to reassociate before being further phosphorylated to TDP. The direct channelling of TdR to TDP does not appear to be a significant route. It is possible that, once phosphorylated, the furanosyl ring with the unnatural L configuration cannot be readily accommodated into the active site of the enzyme due to the steric impedence of the 5'-phospho group. This could explain both the low affinity of L-TMP for the enzyme and the fact that L-TdR, which is readily phosphorylated by the HSV-1 TK, has only a relatively low potency against HSV-1 replication in infected cells (Spadari et al., 1992). It is possible that a similar mechanism could be responsible for the lack of phosphorylation of ACV monophosphate by the HSV-1 TMPK activity.

In conclusion, our results suggest that the active site shared by the HSV-1 TK and TMPK activities can readily accommodate nucleoside analogues with several modifications both in the base and in the sugar ring, but it is not so tolerant in the acceptance of the same analogues once they are phosphorylated in the 5'-position of the sugar ring. These observations could be relevant in designing new antiviral nucleosides.

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