Structure to 1.9 Å resolution of a complex with herpes simplex virus type-1 thymidine kinase of a novel, non-substrate inhibitor: X-ray crystallographic comparison with binding of aciclovir

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Received 13 November 1998; received in revised form 30 November 1998

Abstract Treatment of herpes infections with nucleoside analogues requires as an initial step the activation of the compounds by thymidine kinase. As an aid to developing more effective chemotherapy, both for treatment of recurrent herpes infection and in gene therapy systems where thymidine kinase is expressed, two high-resolution X-ray structures of thymidine kinase have been compared: one with the relatively poor substrate aciclovir (Zovirax), the other with a synthetic inhibitor having an N^2-substituted guanine. Both compounds have similar binding modes in spite of their size difference and apparently distinct ligand properties.

Key words: Herpes simplex; Aciclovir; Thymidine kinase; Drug binding; X-ray crystallography

1. Introduction

Herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) can give rise to painful mouth, corneal or genital ulcers. HSV-1 thymidine kinase (ATP:thymidine 5-phosphotransferase; EC 2.7.1.21) (TK) is the centre of activation of antiviral drugs such as aciclovir (Zovirax) [1,2]. Such nucleoside analogues block the viral replication process, firstly being phosphorylated by virally encoded TK or TK-related enzymes, and subsequently terminating DNA elongation at the viral DNA polymerase. Selectivity is ensured through the inability of the host-cell TK to phosphorylate these drugs to any appreciable extent. These compounds have been employed in viral studies of TK mutations [3], and have also been used in gene therapy for cancer [4,5] to achieve selective toxicity after introduction of the viral TK gene into tumour cells. TK itself is not a direct target for antiviral therapy, because the enzyme is not required for virus replication in proliferating cells. However, viral TK activity may be required for reactivation of virus from latency in nerve cells [6]. TK inhibitors have potential use in treatment of recurrent herpes infections [7].

Whilst it has a good reputation as an antiherpetic drug, aciclovir has rather poor efficacy, being a weak substrate for TK, in spite of the strong chain-terminating effect of its triphosphate. As part of the search for novel TK inhibitors, which could be useful chemotherapeutic agents to prevent recurrent herpes infections, a group of compounds based on *N*-phenyl derivatives of guanine has been developed [8]. Some of these display good competitive inhibition, especially one acyclic nucleoside analogue, 9-(4-hydroxybutyl)-N^2-phenylguanine (HBPG), which does not act as a substrate. HBPG has been found to reduce the frequency of recurrent HSV-1 ocular disease in two animal models [9,10]. Two molecular modelling studies on the binding of N^2-phenylguanines to TK gave differing results, one [11] predicting such compounds to bind in a manner distinct from that found [12,13] for ganciclovir, the other [14] predicting, for HBPG, a similar binding mode.

X-ray studies on a range of HSV-1 TK/ligand complexes to medium and high (2.14 Å) resolutions have been reported [12,13,15,16]. This X-ray study was undertaken under cryocooled conditions at higher resolution (1.9 Å), firstly to determine details of aciclovir binding not clearly characterised to date, and secondly to see if the high affinity and lack of substrate activity of HBPG could be understood in structural terms.

2. Materials and methods

2.1. Crystallisation and data collection of TK/aciclovir and TK/HBPG binary complexes

Expression and purification of residues 11–376 of the complete 376 amino acid thymidine kinase peptide has been described previously [12,17,18]. Crystals of the TK/deoxythymidine (dT) complex were formed by vapour diffusion in which 8 μl of protein solution consisting of 1.0 mg/ml HSV-1 TK, 40 mM Tris-HCl pH 7.5, 3 mM DTT and 0.2 mM deoxythymidine were mixed with 4 μl of precipitating solution (30% saturated ammonium sulphate, 200 mM Tris-HCl pH 6.75, 3 mM DTT and 0.2 mM deoxythymidine), the mixture being equilibrated against the precipitating solution at 25°C. The crystals belong to orthorhombic space group $C222_1$. Exchange of bound deoxythymidine for aciclovir or HBPG [8] was done by soaking crystals five times in 3 ml of solution containing a stoichiometric amount of compound, 33% saturated ammonium sulphate and 100 mM Tris-HCl pH 6.75. Crystals were flash-frozen by transfer to a cryoprotecting solution (33% saturated ammonium sulphate, 100 mM Tris-HCl pH 6.75, 25% glycerol, and compound) before being mounted in a cryostream for in-house X-ray inspection. Cryocooled crystals showing

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0014-5793/99/$19.00
PII: S0014-5793(98)01619-6

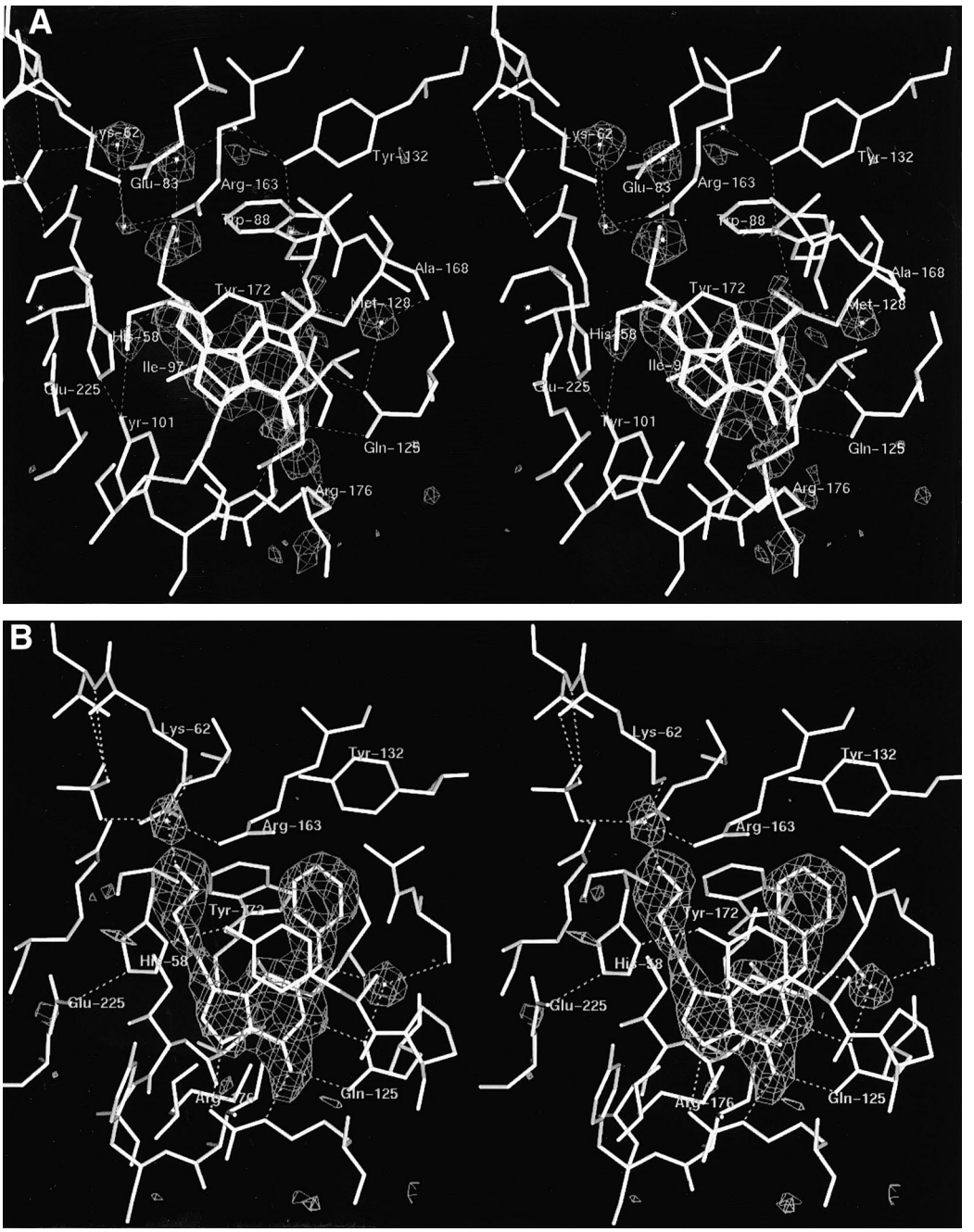

Fig. 1. Stereo view of the binding of (A) aciclovir and (B) HBPG to TK (molecule 1) superimposed on a difference Fourier map contoured at $3\times\sigma$ density and showing intermolecular hydrogen bonds. Owing to ambiguity in the density for the hydroxyethoxymethyl group of aciclovir (see text), two possible locations for the group are indicated in A.

diffraction to at least 1.9 Å resolution were taken to the 6 GeV synchrotron source at ESRF Grenoble using an in-house system [19] consisting of a portable cold nitrogen stream which allows crystals to be transferred from goniometer to a storage vessel and back to a goniometer. Full three-dimensional data were collected at ESRF (station BM14) on a Mar345 imaging plate. Data from 90° oscillation range (0.8° per plate) were processed with DENZO/SCALEPACK [20], and TRUNCATE from the CCP4 suite [21]. Statistics of data processing are shown in Table 1A.

2.2. Refinement of molecular models of complexes

Co-ordinates of the latest best TK/dT model [13] (with ligand and active-site water molecules excluded) were used as a starting point for refinement of atomic positions and temperature factors against data from the TK/aciclovir and TK/HBPG complexes. Refinement and difference Fourier electron density map calculation were done using X-PLOR [22,23], as described previously [13]. Modelling was done on an Evans and Sutherland Freedom workstation using the program O [24]. Refinement statistics are given in Table 1B. Co-ordinates will be deposited with the Protein Data Bank at Brookhaven.

3. Results

The structure of the TK/aciclovir complex, previously solved to 2.37 Å resolution, has now been extended to 1.9 Å through use of a high-energy synchrotron source. The new complex of TK/HBPG was similarly examined to 1.9 Å resolution. Both sets of data were collected from cryocooled

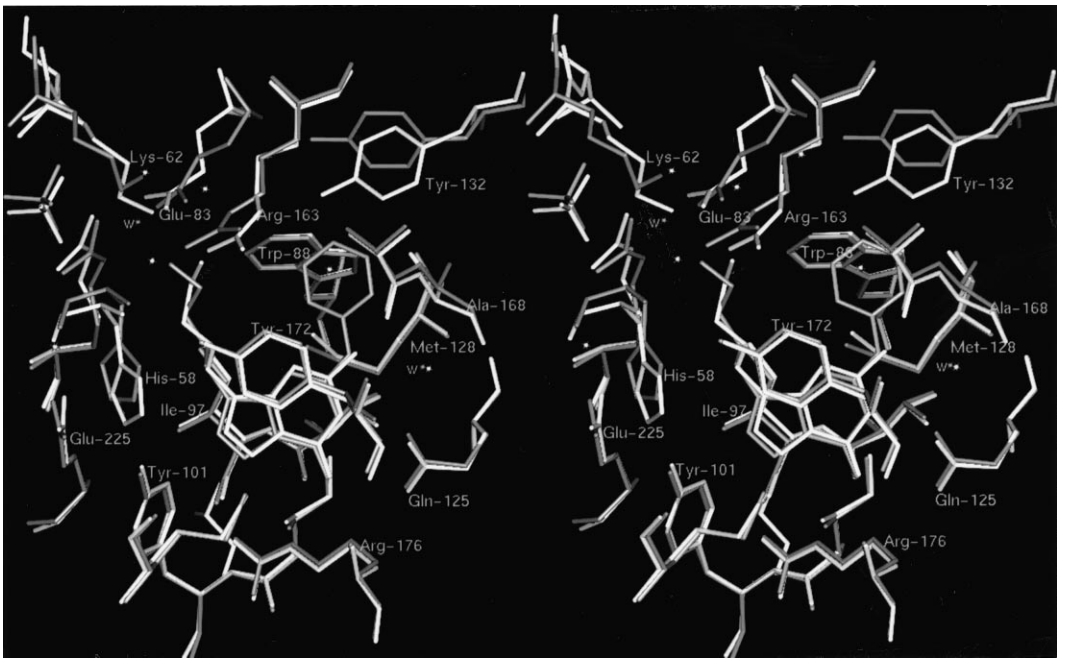

Fig. 2. Stereo view of the molecule 1 active site of the TK/HBPG complex (grey) overlaid on the active site of the TK/aciclovir complex (white) after alignment of enzyme molecules. (Water molecules in the HBPG complex are labelled 'w'.)

crystals, a novel method [19] having been used for transportation of such crystals following initial in-house evaluation. The overall fold of the enzyme, and binding of several nucleoside analogues have been described previously [13]. There are two molecules in the asymmetric unit.

3.1. TK/aciclovir complex

In the earlier aciclovir structure determination [13] it was possible to make a structural interpretation of the difference density for molecule 1 only. The quality of difference density for molecule 2 is improved with the new high-resolution data, and is now interpretable. The binding mode observed for molecules 1 and 2 broadly confirms the location of the guanine moiety previously reported for molecule 1[13]. In respect of the hydroxyethoxymethyl chain, density for molecule 2 appears to extend so that the chain can be modelled with a hydroxyl location mimicking the location of the 5′-hydroxyl group of the substrate dT. Although this is in the region of the side chain of Glu-83 and nearby waters, all are too distant to be modelled with hydrogen bonds. The unbranched chain cannot also mimic the 3′-hydroxyl location, near to the Tyr-101 side chain. By contrast, for molecule 1, while there is also, albeit detached, density close to the 5′-hydroxyl position (representing structure which can interact via hydrogen bonds with Lys-62, Glu-83, Asp-162 and Arg-163), difference density continuous at the $2.5\times\sigma$ level suggests an equally likely interpretation in terms of a 3′-hydroxyl mimic, which would make a hydrogen bond to the hydroxyl group of Tyr-101 (Fig. 1A). This suggests the viability of alternative conformations for the molecule 1 hydroxyethoxymethyl group, both

Table 1
X-ray statistics for thymidine kinase/ligand complexes

A: X-ray reflection-data statistics

Ligand	$C222_1$ unit cell (Å)			Resolution (Å)	R_{sym}	Unique data	Completeness	
	a	*b*	*c*				All data	Outer shell
Aciclovir	114.1	117.8	108.8	1.82	0.045	63 458	97%	57%
HBPG	113.4	118.0	109.0	1.87	0.044	55 222	92%	41%

B: Refinement of molecular models

Ligand	No. of data	Resolution (Å)	Completeness (σ_{min})	*R* value (R_{free})	No. of waters	RMS bond (Å)	Mean *B* (Å^2)
Aciclovir	47 727	1.90	83% (3.0)	0.241 (0.317)	292	0.012	22.5
HBPG	49 954	1.90	89% (3.0)	0.225 (0.289)	293	0.012	18.8

A: Statistics from Denzo/Scalepack processing [20]. $R_{sym}=\Sigma|I_i-\langle I\rangle|/\Sigma I_i$; where I_i is the measured intensity of an individual reflection, and $\langle I\rangle$ the mean of repeated measurements. Completeness for outer shell gives percentage of data in resolution range 2.00–1.93 Å with $I>2.0\times\sigma(I)$.
B: The number of X-ray data are those extending to the resolution stated and for which $\sigma>\sigma_{min}$. *R* value is a measure of the agreement between calculated and observed X-ray data: $R=\Sigma||F_{obs}|-|F_{calc}||/\Sigma|F_{obs}|$ where F_{obs} and F_{calc} are the observed and calculated structure factors respectively. R_{free} is similarly defined for a test set (10%) of the reflections excluded from driving refinement. RMS bond (X-PLOR software) is a measure of the discrepancy between refined and standard bond lenghts. Mean *B* is the average temperature factor of main chain atoms after two pairs of positional and temperature-factor refinement rounds. The mean *B* for aciclovir and HBPK non-hydrogen atoms are 27.4 and 15.1 Å^2 respectively.

having comparable binding enthalpy and both present in the crystal. For the guanine moiety, there is a water molecule hydrogen-bonded to both the 2-NH_2 group and the side chain carbonyl of Gln-125. Gln-125 forms the classical pair of hydrogen bonds with ligand groups 1NH and O6. The positions of the guanine group and Arg-176 side chain indicate an absence of strong hydrogen bonding between aciclovir and Arg-176 (the same result was obtained for ganciclovir [13]), although, in molecule 1, the ε-NH_2 group and ligand O6 are close (3.3 Å); for molecule 2 (not shown), difference density suggests indirect hydrogen bonding for these atoms, via a water molecule.

3.2. TK/HBPG complex

The difference density for this compound is unambiguous for both molecules of the asymmetric unit (Fig. 1B), and the binding mode is essentially the same for both. The unbranched hydroxybutyl chain, an isostere of the corresponding group in aciclovir, interacts with the carboxylate of Glu-83 and, via a water molecule, with the side chains of Lys-62 and Arg-163. This mimics the location and interactions of the 5′-hydroxyl of substrate dT [13]. The binding location of the guanine portion of this ligand is essentially the same as described above for aciclovir with the slight difference that the larger HBPG is constrained into closer and more numerous contacts with surrounding active site atoms; for example, the N7 and O6 atoms of guanine are clearly in hydrogen bond range of the guanidinium of Arg-176. Gln-125 side chain interacts as expected with N1 and O6 of guanine. The N^2-phenyl group is in van der Waals contact with non-polar atoms of several residues (Tyr-132, Arg-163 and Ala-168 have atoms within 3.5 Å, and Trp-88, Met-128 and Tyr-172 also have atoms nearby); the 2-NH group interacts via a water molecule with polar groups of Gln-125 and Ala-168. The torsion angle pairs for the N^2-phenyl moieties of molecules 1 and 2 are (180°,30°) and (−170°,10°) respectively.

4. Discussion

We have now clarified that aciclovir binding (for both molecules in the asymmetric unit) is similar to that reported for ganciclovir and penciclovir [13]. Like ganciclovir [13], aciclovir does not completely fill the active site of TK. Unlike dT and uracil analogues [13,25], the base moiety does not readily form a double hydrogen bond with the side chain of Arg-176. However, despite repeated attempts to obtain unambiguous information, a single mode of binding for aciclovir's hydroxyethoxymethyl group is still not clearly established. It is therefore likely that the difference density maps highlight a real ambiguity: the presence of two binding modes for the group of comparable binding energy, one locating the hydroxyl in the 5′ position of substrate dT, the other in the 3′ position. Presumably the presence of only one hydroxyl group with which to mimic the 3′- or 5′-hydroxyl groups of dT contributes to aciclovir's weak binding.

By contrast HBPG binds strongly to TK [8]. Whilst, like aciclovir, HBPG has an unbranched acyclic hydroxyl-bearing group, it nevertheless shows an unambiguous binding mode for the group. The N^2-phenyl group is probably a decisive factor in the stronger binding. The presence of the group appears to constrain the guanine into closer proximity with Arg-176, the ligand forming hydrogen bonds with this residue. The N^2-phenyl group makes a good van der Waals fit in the cavity near Tyr-132, a location that can also be occupied by 5-substituents of tightly binding uracil analogues [13]. The binding modes of aciclovir and HBPG are compared in Fig. 2.

One theoretical study of complexes of N^2-phenylguanines with TK predicted binding to TK in a manner distinct from that found for the structurally related drug molecule ganciclovir [11]. In another [14] the binding mode of the guanine of HBPG was predicted to be similar, as now confirmed by this X-ray study. As HBPG, by contrast with the drug molecules, acts as an inhibitor rather than as substrate [8], the intriguing question remains as to what is the structural basis for the different properties. Given that the HBPG's presentation is similar to that of the weak substrate aciclovir, and its potential phosphate-binding function (the hydroxybutyl group) is isosteric with aciclovir's, it would appear that its inhibitory properties arise from the much greater binding enthalpy that the van der Waals interactions of the additional N^2-phenyl group give rise to. The high affinity, arising, no doubt, from these interactions, may restrict the flexibility of the complex to allow phosphorylation of the ligand. It is also possible that V_{max} is sufficiently low, even compared to that of aciclovir, that no phosphorylation of HBPG has been observed under assay conditions [8].

Acknowledgements: We thank Dr M. Sohi for expert advice and technical assistance, Dr G. Leonard and Dr A. Thompson of ESRF, Grenoble, for their advice on the use of BM14, and Mrs C.R. Kirwan for assistance with illustrations. The work was supported by the Wellcome Trust, and also in part by SBIR Grant AI43170 from the National Institutes of Health (to G.E.W.).

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