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Database Searching for Thymidine and Thymidylate Kinase Inhibitors Using Three-dimensional Structure-based Methods

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Structure-based drug design methods were used to search for novel inhibitors of herpes simplex virus type 1 (HSV-1) thymidine kinase and *Mycobacterium tuberculosis* thymidylate kinase. The method involved the use of crystal structure complexes to guide database searching for potential inhibitors. A number of weak inhibitors of HSV-2 were identified, one of which was found to inhibit HSV-1 TK and HSV-1 TK-deficient viral strains. Each compound tested against *M. tuberculosis* thymidylate kinase was found to have some activity. The best of these compounds was only 4.6-fold less potent than 3′-azido-3′-deoxythymidine-5′-monophosphate (AZTMP). This study demonstrates the utility of structure-based drug design methods in the search for novel enzyme inhibitors.

Keywords: Thymidine kinase inhibitors; Database searching; Structure-based drug design; Thymidylate kinase; Herpes simplex virus type 1; *Mycobacterium tuberculosis*

INTRODUCTION

At least seven herpes viruses use humans as their host giving rise to a number of painful conditions. Herpes simplex virus type 1 (HSV-1) commonly erupts as epithelial ulcers in the upper part of the body such as the mouth or the eyes. HSV-2 on the other hand, usually presents itself as ulcers to the genitals and is transmitted sexually.[1] The antiviral drug aciclovir is activated by HSV-1 thymidine kinase (ATP: thymidine 5-phosphotransferase; E.C. 2.7.1.21) (TK).[2,3] When the activated form of the drug is incorporated into the viral DNA this terminates DNA synthesis.[4,5] While aciclovir is effective, it is considered to be a poor substrate for TK and there is a need for novel chemotherapeutic agents with improved profiles.[6]

During the last 20 years the incidence of tuberculosis (TB) has risen steadily.[7] The organism, *Mycobacterium tuberculosis*, is transmitted through aerosolised secretions and may lie quiescent for many decades. This can be particularly problematic for patients with HIV infections. Resistant strains have also emerged possibly as a consequence of patients not completing their therapies which usually consists of a number of agents taken for at least 6 months.[7] There is a clear need for new antibiotics and a potential target for these agents is the enzyme thymidine monophosphate kinase (E.C. 2.7.4.9, $TMPK_{Mtub}$),[8] which catalyses the phosphorylation of thymidine monophosphate to thymidine diphosphate. The crystal structure of $TMPK_{Mtub}$ has recently been solved (Protein Databank[9] (pdb) code 1g3u)[10,11] and was employed in this study to find novel inhibitors.

This study has explored the use of structure-based drug design techniques to generate search queries to find potential TK inhibitors for HSV-1 and TMPK inhibitors for TB.

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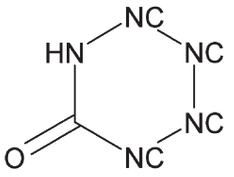

FIGURE 1 Substructure used to identify suitable search candidates from the ACD[13] and SPECS & BioSPECS[17] databases. The search was aimed at finding six membered heterocyles where NC refers to atoms in this position being nitrogen or carbon. The bonds between the NC atoms were designated as "any" bonds meaning they could be single, double or aromatic.

MATERIALS AND METHODS

Molecular Modelling and Database Searching

From a survey of various herpes simplex virus (HSV) thymidine kinase crystal structures complexed with inhibitors, it was observed that two DNA bases have the ability to bind to the active site (thymine and guanine).[12] The searches described in this study have used the crystal structure complex of a thymine analogue as a guide, pdb code 1kim.[12] While guanine analogues were of interest, we opted to create our search queries based on the natural base (thymine) for this enzyme.

The Available Chemicals Directory[13] (ACD) was used as a database of potential screening compounds. An initial filter was applied to the database by selecting compounds that had a molecular weight under 800 and matched the substructure shown in Fig. 1.

Using the UNITY package,[14] a search query was constructed seeking molecules containing the following molecular fragments:

a. A six membered heterocycle (Fig. 1) located in the position of the thymine ring of the 2′-deoxythymidine inhibitor complexed in 1kim.[12]
b. An atom based on the location of atom *x* (Fig. 2a) of the 2′-deoxy thymidine structure in 1kim. This atom was chosen as it lies deep within the binding pocket. The aim of the search was to select structures that not only possess an appropriate six membered heterocycle but also have a group that may occupy the remainder of the binding site. This atom could be any of the following atom types: N, C, S, O, F, Cl or Br.
c. Similarly, an atom located 2.5 Å from atom *y* (again using the co-ordinates of the 2′-deoxythymidine inhibitor in 1kim to guide the location) along the *y*–*z* vector (Fig. 2a). This atom was defined as N, C, S, O, F, Cl or Br. The same reasoning of trying to find ligands that occupy active site space was applied to define this atom location. The distance of 2.5 Å was chosen to avoid the selection of smaller substituents.

To allow flexibility in the location of the three molecular fragments, spatial points were placed on specific atoms. In this case, atoms 1–6 were assigned precise spatial positions and spheres of radius 0.3 Å for atoms 1–4, 0.6 Å for atom 5 and 1.25 Å for atom 6 were placed in these locations (Fig. 2b). For the search itself, the default parameters for flexible searches within UNITY[14,15] were used which controls conformational flexibility, allocates 60 s search time per molecule, checks for internal VDW bumps and allows a limited planar tolerance for amide bonds. To mimic the binding site cavity, occlusion spheres (radius 0.6 Å) were generated on each heavy atom in the protein located within 5 Å of the 2′-deoxythymidine structure in 1kim.

Following the database search the following filters were applied to the list of selected compounds to reduce it further. To a large extent, these assessments were qualitative.

a. Novelty (largely distinct in structure from known inhibitors).
b. Chemical stability/reactivity (human assessment).
c. Visual appraisal of each compound in the binding site looking for the potential to hydrogen bond and space-filling characteristics.

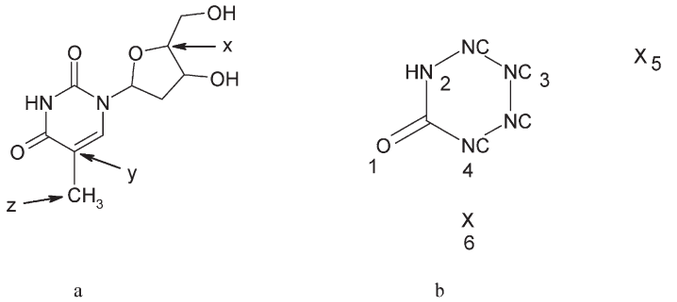

FIGURE 2 (a) Structure of 2′-deoxythymidine indicating the atoms used in defining the database search. (b) Diagram showing the three molecular fragments defined for the database search and atom numbering used to define spatial points.

TABLE I Inhibition constants (K_i^{app}) and solubility data for the $TMPK_{Mtub}$ assay

Compound	Solubility conditions of stock solutions (5–10 mM)	K_i^{app} (μM)
1	60% Isopropanol in Buffer A	570
2	Buffer A* (pH 9)	670
3	Buffer A (pH 9)	750
4	Dioxane (100%)	320
5	60% Isopropanol in Buffer A	670
6	60% Isopropanol in Buffer A	750
7	60% Formamide in Buffer A	230
AZTMP	Buffer A (pH 9)	50

*Buffer A consisted of Tris–HCl 50 mM pH 9.0, 20 mM Magnesium acetate, 100 mM KCl, 1 mM EDTA and 0.5 mM DTT.

d. Price/availability/known pharmacology using the ACD database information.

Ideally, the application of a method that both docks and scores compounds bound into the active site of TK enzymes automatically would be preferred to prioritise purchasing decisions. Unfortunately, following the examination of a well-studied series of *N*-phenylguanine based TK inhibitors[16] we were unable to develop a suitable model to rank this set of compounds (results not shown). Therefore, visual inspection was deemed to be a preferable method to select screening candidates.

For $TMPK_{Mtub}$ the same search strategy was applied to that outlined for 1kim above. In this case, the collection of compounds available from SPECS and BioSPECS[17] was searched as it provided an opportunity to increase the diversity of the final screening set.

Docking Studies

Selected compounds from the $TMPK_{Mtub}$ screen were further analysed using molecular docking methods. This technique provides valuable insights into the potential binding mode of a ligand, which can assist in lead optimisation. Compounds were docked into the crystal structure of $TMPK_{Mtub}$[11] using the program GOLD[18] applying the default parameters on a Silicon Graphics Octane 2 workstation.

Enzyme and Cellular Assays

Mycobacterium tuberculosis

The $TMPK_{Mtub}$ protein used in this study was overexpressed in *E. coli* and purified as described by Munier-Lehmann and co-workers.[8] Given problems with the solubility of a number of the test compounds, a variety of conditions were used to get them into solution. Typically, slightly alkaline conditions increased their solubility and a buffer of pH 9.0 was employed (Table I). The pH optimum of $TMPK_{Mtub}$ activity was 7.5 (unpublished results), and at pH 9.0 the enzyme retained 40% of its maximal activity.

Kinase activity was directly measured by HPLC chromatography as described below. In our case a coupled spectrophotomeric assay[19] was not used as many of the test compounds absorbed light at 340 nm thus making it impractical to evaluate the concentration of NADH in the coupled reaction. The enzymatic coupling test may also be problematic due to (i) the high pH value of buffer A, (ii) the possibility of recycling of both substrate or product during reaction coupling.[20] As such, errors may be incurred in the determination of K_i and K_m values. Nevertheless, to validate the HPLC assay, both ATP and TMP were tested using the direct method and reaction coupling[19] to demonstrate that their apparent Michaelis constants were the same.

The enzymatic reaction (1 ml final volume) was carried out in buffer A: Tris–HCl 50 mM pH 9.0, 20 mM Magnesium acetate, 100 mM KCl, 1 mM EDTA, 0.5 mM DTT, in the presence of different concentrations of ATP and TMP, with or without inhibitors. To follow the enzymatic kinetics an aliquot (100 μl) of the reaction medium was taken at different times and the reaction quenched by adding 900 μl of sodium phosphate 100 mM pH 7. Changes in the concentration of each nucleotide (ATP, ADP, TMP and TDP) at different times during the reaction was measured at 260 nm by HPLC chromatography (isocratic mode) using a Sephasil™ C18, 5 μm SC 2.1/10 (Amersham Pharmacia) column. All the reaction velocities were calculated by monitoring the production of TDP expressed in terms of optical absorbance per minute at 260 nm.

In our hands, the catalysis reaction of $TMPK_{Mtub}$ followed a Michaelis model. The initial velocity of the reaction at a fixed saturating concentration of ATP and different concentrations of TMP in the absence and in the presence of inhibitors allowed the determination of the apparent Michaelis constant for TMP. Indeed, when the concentration of enzyme is much smaller than the substrate concentration, the initial velocity of the reaction for each substrate concentration can be taken as the slope of the initial linear portion of each kinetic reaction (Fig. 3). In the case of a competitive inhibitor,[21] using the Lineweaver-Burk representation, the following equation applies:

$$K'_m = K_m\left(1 + \frac{[I]}{K_i}\right)$$

where K'_m and K_m are the apparent Michaelis constants determined in the presence and the absence of inhibitor [I], respectively. Knowing the concentration of the inhibitor, it is possible to

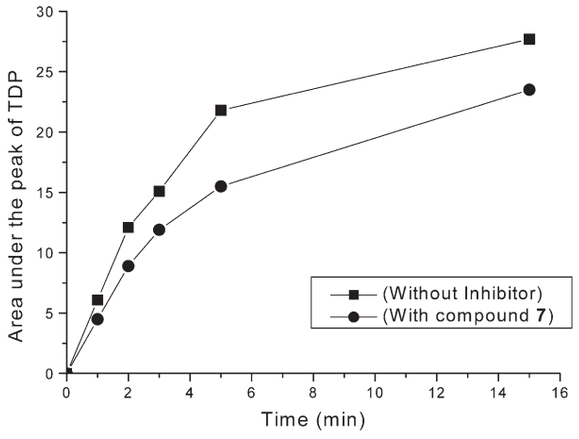

FIGURE 3 Diagram plotting the TDP concentration against time during the enzymatic reaction with and without compound **7**. The initial concentration of ATP and TMP was 1 and 0.380 mM, respectively. The concentration of the enzyme $TMPK_{Mtub}$ was 5×10^{-8} M and the concentration of compound **7** was 600 μM.

calculate K_i^{app} values (at a fixed concentration of ATP).

Herpes Simplex

The 50% inhibitory concentration (IC_{50}) for the test compounds against phosphorylation of [CH_3–^{3}H] dThd as the natural substrate by purified recombinant HSV-1 TK and HSV-2 TK (prepared and purified according to a previously published procedure[22]) was determined under the following reaction conditions: the standard reaction mixture (50 μl) contained 50 mM Tris–HCl, pH 8.0, 2.5 mM $MgCl_2$, 10 mM dithiothreitol, 2.5 mM ATP, 10 mM NaF, 1.0 mg/ml bovine serum albumin, 1 μM [CH_3–^{3}H]dThd (0.1 μCi), an appropriate amount of test compound and 5 μl milli Q water. The reaction was started by the addition of enzyme, incubated at 37°C for 30 min, and the reaction was terminated by spotting an aliquot of 45 μl onto DE-81 discs (Whatman, Maidstone, UK). After 15 min, the discs were washed for 3 times at 5 min in 1 mM $HCOONH_4$ while shaking, followed by 5 min in ethanol (70%). Finally, the filters were dried and assayed for radioactivity in a toluene-based scintillant. The IC_{50} was defined as the drug concentration required to inhibit thymidine phosphorylation by 50%.

Antiviral activity and cytotoxicity assays with selected test compounds were carried out using E_6SM cell cultures. Visual inspection of cells was undertaken according to previously published procedures[23] using various concentrations of test compound looking for changes to cell morphology, which indicate cytotoxic activity. Antiviral activity was determined using various viral strains including TK deficient forms and was expressed as the concentration needed to reduce virus-induced cytopathogenicity by 50%. The test compound was compared with a number of standard antiviral agents.

RESULTS AND DISCUSSION

Mycobacterium tuberculosis

Using the $TMPK_{Mtub}$ crystal structure, the list of 196 compounds generated from the database search was trimmed to 14 compounds. Nine of these compounds were available and obtained for screening purposes. Unfortunately, two of these compounds were found to be insoluble and were not tested as enzyme inhibitors. In the assay employed in this study, a decrease in the TMP and ATP concentrations and a concomitant increase in the TDP and ADP concentrations occurs during the incubation. By following the production of TDP, the inhibition of $TMPK_{Mtub}$ could be determined for the seven test compounds (compounds **1**–**7**, Fig. 4) and 3′-azido-3′-deoxythymidine-5′-monophosphate (AZTMP) (Table I). An example plot showing the initial velocity of the reaction for compound **7** is shown in Fig. 3. Our experiments demonstrated that all the compounds tested were competitive with TMP (see e.g. Figs. 5 and 6). While each compound was shown to have some activity against the enzyme (Table I), only two of these compounds (**4** and **7**) demonstrated reasonable affinity. Compound **7** was only 4.6-fold less potent than the standard, AZTMP, representing a novel inhibitor for this enzyme. While the other compounds inhibited enzyme activity, they were of less interest as potential leads against TB.

Both compounds **4** and **7** were docked into the crystal structure of $TMPK_{Mtub}$. In each case, the thymine-based ring systems overlap fairly well with that of TMP in the crystal structure,[11] as expected. From this docking work compounds **4** and **7** were predicted to form two hydrogen bonds to arginine 74 where TMP only forms one bifurcated hydrogen bond (Fig. 7). For compound **4**, the furanyl oxygen is predicted to bind in the same location as the 3′ hydroxyl oxygen of TMP. While for compound **7**, the terminal carbon of the ethoxyl group occupies this position.

As highlighted by Li de la Sierra *et al.*,[11] two residues in the TMP binding site of $TMPK_{Mtub}$ stand out as being different from those of orthologous enzymes and could therefore be exploited in the design of inhibitors selective for *M. tuberculosis*. The first of these residues, asparagine 100, is predicted to form a hydrogen bond with both compounds **4** and **7**. As such, it is difficult to see how simple analogues of these compounds could be made that would exploit this interaction further. The hydroxy

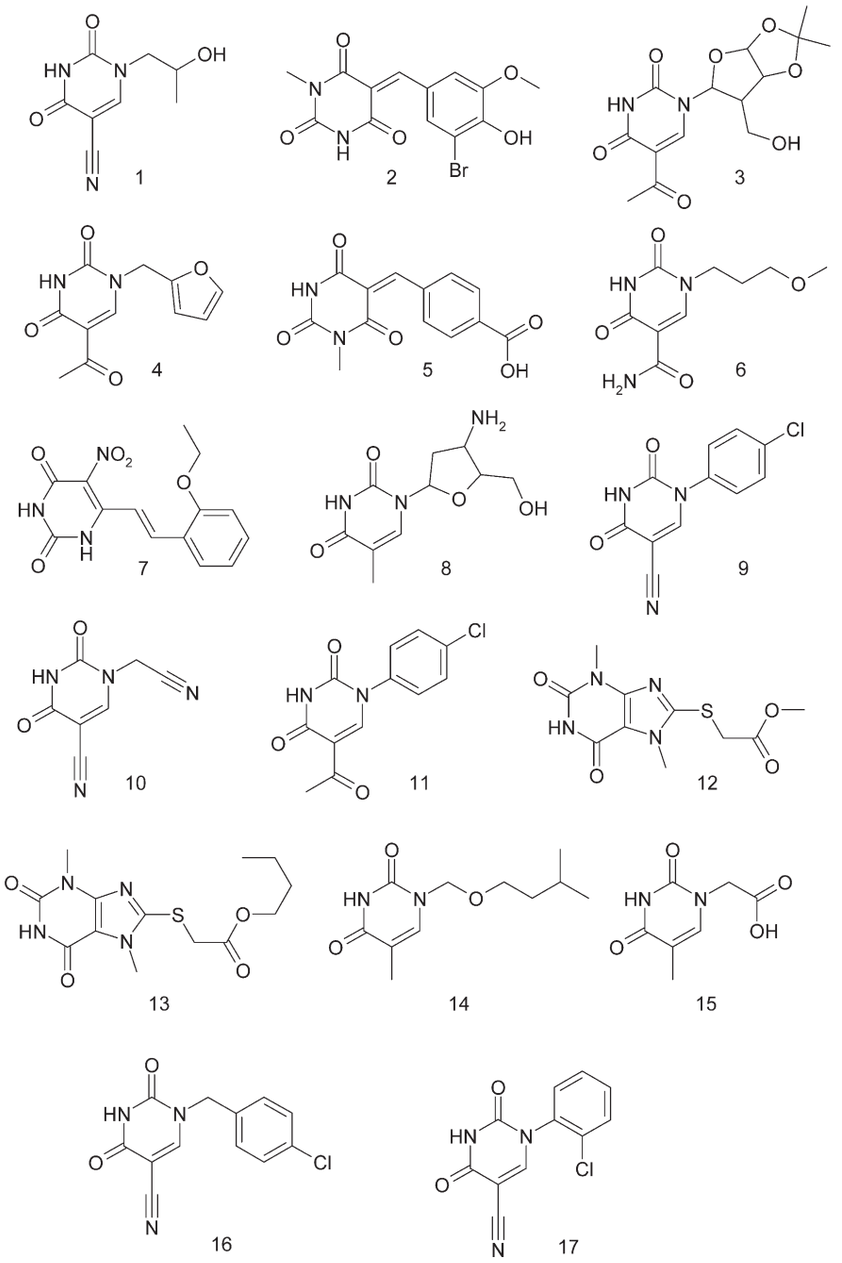

FIGURE 4 Chart of the structures used in this study.

group of the second residue, tyrosine 39, is known to form a hydrogen bond (O–O distance, 3.1 Å) with the phosphonyl oxygen atom of TMP.[11] The carbon atom at position 4 of the furanyl ring of **4** is predicted to lie 4 Å from the hydroxyl oxygen of this residue. Substitution at the 4 position could possibly lead to compounds selective for $TMPK_{Mtub}$. The phenyl ring of **7** is also predicted to lie close to the phenol oxygen of Tyr39 and is orientated so that the two rings are perpendicular. The distance from the centroid of the phenyl ring of compound **7** to the phenol oxygen of Tyr39 is 3.4 Å. While it is not possible to envisage a substitution pattern that could directly interact with Tyr39, one may be able to influence the electronic nature of the phenyl ring of compound **7** to enhance potency.

Herpes Simplex

The list of 170 compounds identified from the database search using the crystal structure of HSV TK (1kim) was trimmed to 20 screening candidates.

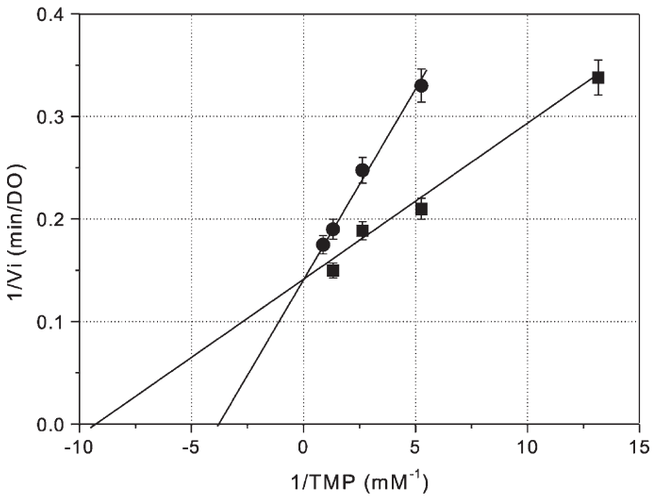

FIGURE 5 Lineweaver-Burk plots for $TMPK_{Mtub}$ tested in the absence (■) and presence (●) of compound **4** (375 μM). Experiments were conducted in Buffer A with the inhibitor diluted 10 times from the stock solution defined in Table I and the concentration of $TMPK_{Mtub}$ was 5×10^{-8} M; ATP was in large excess (1 mM). Experiments conducted in the absence of inhibitor were made using the same solvent conditions as those containing the inhibitor (i.e. approximately 10% of organic solvent was present in the reaction medium).

Of these only ten were available from commercial suppliers (compounds **8**–**17**, Fig. 4). Each compound was purchased and subsequently studied for enzyme inhibitory activity against HSV-1 and HSV-2 TK (Table II).

Compound **8** demonstrated reasonable activity in the HSV-1 TK assay and was considered to be an interesting hit. In addition, this compound also showed good activity in the HSV-2 TK assay. Indeed, compounds **9**, **11**, **13**, **14** and **16** also showed moderate to weak inhibition of HSV-2 TK. While we were not seeking activity against HSV-2 TK, these serendipitous findings may spawn additional studies.

As compound **8** showed activity in both TK assays, further studies were conducted in order to help elucidate its mechanism of action. This included the testing of known standards and evaluations against other viruses to investigate selectivity issues (Table III).

While compound **8** demonstrated activity in the HSV-1, HSV-2 and vaccinia virus assays, activity was also demonstrated against the TK deficient HSV-1 strains. This suggests that compound **8** is acting

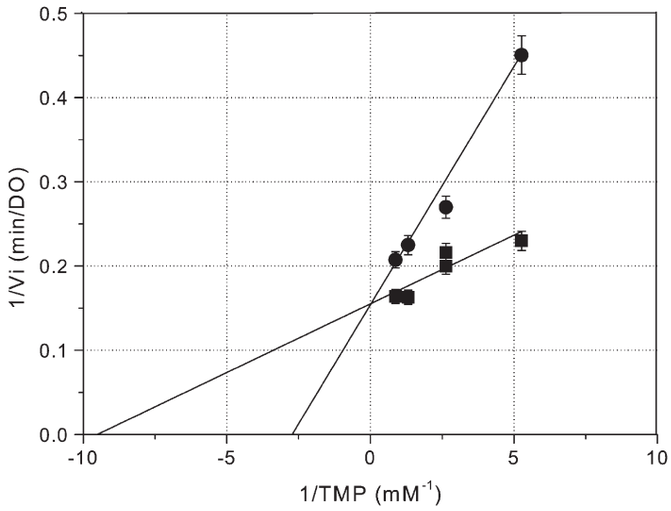

FIGURE 6 Lineweaver-Burk plots for $TMPK_{Mtub}$ tested in the absence (■) and presence (●) of compound **7** (600 μM). Experiments were conducted using Buffer A with the inhibitor diluted 10 times from the stock solution defined in Table I and the concentration of $TMPK_{Mtub}$ was 5×10^{-8} M; ATP was in large excess (1 mM). Experiments conducted in the absence of inhibitor were made using the same solvent conditions as those containing the inhibitor (i.e. approximately 6% of organic solvent was present in the reaction medium).

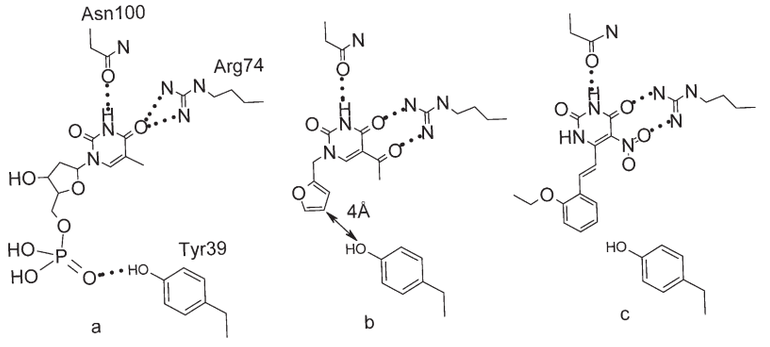

FIGURE 7 Comparison of the binding mode of TMP (a) in the $TMPK_{Mtub}$ crystal structure[11] and the predicted binding modes of compounds **4** (b) and **7** (c). Hydrogen bonds are indicated with dashed lines.

TABLE II HSV-1 and HSV-2 thymidine kinase inhibition

Compound	HSV-1 TK	HSV-2 TK
8	5.2 ± 0.02	16.4 ± 6.2
9	>200 (36%)	72.1 ± 7.7
10	>200 (15%)	>200 (25%)
11	>200 (41%)	121 ± 28
12	>200 (38%)	>200 (34%)
13	>200 (35%)	135
14	>200 (24%)	166 ± 41
15	>200 (0%)	>200 (6%)
16	>200 (19%)	157 ± 14
17	>200 (6%)	>200 (32%)

50% inhibitory concentrations given in μg/ml. Percentage inhibition at 200 μg/ml is indicated in parentheses. Results are given as the mean ± standard error of at least two determinations.

through a mechanism other than TK inhibition. Interestingly, this compound has been examined previously against both HIV-1[24] and Hepatitis B virus,[25] but did not demonstrate any antiviral activity. Compound **8** is a known cytotoxic agent and is thought to act via the inhibition of DNA polymerase.[26] This cytotoxic activity may have interfered with the antiviral assays, although the concentration observed to inhibit viral-induced cytopathogenicity was less than that required to alter normal cell morphology (Table III). Despite this observation, further interest in this compound from a development perspective would be limited as it is a known cytotoxic agent. From an experimental viewpoint compound **8** remains, however, an interesting antiviral agent whose mechanism of action has not been fully elucidated.

This study has demonstrated the utility of structure-based drug design methods in finding novel anti-infective agents. It could be envisaged that the molecules emerging from this study represent the first steps in a medicinal chemistry lead optimisation campaign to develop therapeutic agents against HSV-1, HSV-2 and TB. Indeed, preliminary docking experiments have suggested potential binding modes for two of the compounds that inhibit $TMPK_{Mtub}$. This information was used to suggest modifications to both hit molecules that could potentially lead to compounds with improved affinity and selectivity for $TMPK_{Mtub}$. Another interesting finding from this work, albeit through serendipity, was the identification of a number of compounds which have encouraging inhibitory profiles against HSV-2 TK.

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TABLE III Cytotoxicity and antiviral evaluation results in E_6SM cell cultures

Compound	Minimum cytotoxic concentration* (μg/ml)	Minimum inhibitory concentration (μg/ml)†			
		HSV-1 (KOS)	HSV-2 (G)	Vaccinia virus	HSV-1 TK^- KOS ACV^r
8	>400	9.6	48	9.6	48
BVDU	400	0.016	>80	0.128	>80
Ribavirin	>400	240	80	48	240
ACV	400	0.077	0.077	>80	48
DHPG	>100	0.004	0.004	60	0.8

*Required to cause a microscopically detectable alteration of normal cell morphology. †Required to reduce virus-induced cytopathicity by 50%. BVDU: (E)-5-(2-bromovinyl)-2′-deoxyuridine; ACV: aciclovir; DHPG: 9-[(1,3)-dihydroxy-2-propoxy)methyl]guanine, ganciclovir. The thymidine kinase deficient strain (column 6) represents a drug-resistant (ACV) virus strain.

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