Kinetic analysis of novel multisubstrate analogue inhibitors of thymidine phosphorylase

Jan Balzarini[a,*], Bart Degrève[a], Antonio Esteban-Gamboa[b], Robert Esnouf[c], Erik De Clercq[a], Yves Engelborghs[d], Maria-José Camarasa[b], Maria-Jesús Pérez-Pérez[b]

[a] *Rega Institute for Medical Research, Katholieke Universiteit Leuven, Minderbroederstraat 10, B-3000 Leuven, Belgium*
[b] *Instituto de Quimica Médica, C.S.I.C., 28006 Madrid, Spain*
[c] *Structural Biology Division, Wellcome Trust Centre for Human Genetics, Oxford OX3 7BN, UK*
[d] *Laboratory of Chemistry and Biological Dynamics, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium*

Received 15 August 2000; accepted 21 September 2000

Edited by Pierre Jolles

Abstract A kinetic analysis was performed for the novel 1-(8-phosphonooctyl)-6-amino-5-bromouracil and 1-(8-phosphonooctyl)-7-deazaxanthine inhibitors of *Escherichia coli* thymidine (dThd) phosphorylase (TPase). The structure of the compounds was rationally designed based on the available crystal structure coordinates of bacterial TPase. These inhibitors reversibly inhibited TPase. Kinetic analysis revealed that the compounds inhibited TPase in a purely competitive or mixed fashion not only when dThd, but also when inorganic phosphate (Pi), was used as the variable substrate. In contrast, the free bases 6-amino-5-bromouracil and 7-deazaxanthine behaved as non-competitive inhibitors of the enzyme in the presence of variable Pi concentrations while being competitive or mixed with respect to thymine as the natural substrate. Our kinetic data thus revealed that the novel 1-(8-phosphonooctyl)pyrimidine/purine derivatives are able to function as multisubstrate inhibitors of TPase, interfering at two different sites (dThd(Thy)- and phosphate-binding site) of the enzyme. To our knowledge, the described compounds represent the first type of such multisubstrate analogue inhibitors of TPase; they should be considered as lead compounds for the development of mechanistically novel type of TPase inhibitors.

Key words: Thymidine phosphorylase; Multisubstrate inhibitors; Nucleotide analogues

1. Introduction

Thymidine phosphorylase (TPase) is one of the key enzymes involved in salvage biosynthesis and catabolism of pyrimidine 2′-deoxyribonucleosides. It recognises 2′-deoxythymidine (dThd) and 2′-deoxyuridine (dUrd) as a natural substrate [1]. TPase functions as a dimer of two identical subunits with a dimer molecular mass ranging from 90 kDa (*Escherichia coli*) to 110 kDa (mammals) and catalyses the reversible phosphorolysis of dThd (and other pyrimidine 2′-deoxyribonucleoside analogues) [1]:

pyrimidine 2′ − deoxyriboside + phosphate ⇔

2 − deoxyribose − 1 − phosphate + pyrimidine

TPase also has deoxyribosyl transferase activity between different pyrimidine bases [1]:

pyrimidine 2′ − deoxyribose + *pyrimidine* ⇔ pyrimidine

+*pyrimidine* 2′ − deoxyribose

The structure of human TPase has not yet been determined. However, structural determinations have been reported for two bacterial homologues: *E. coli* [2,3] and *Bacillus stearothermophilus* [4], the latter to a higher resolution. These enzymes have 40% sequence identity with human TPase. Very recently, a model for human TPase has been built based on the crystal structure of *E. coli* TPase, suggesting almost no differences in the main tertiary structure. The TPase structures have revealed that each subunit contains a large mixed α/β domain separated from a smaller α-helical domain by a cleft which contains the substrate-binding sites. In the phosphorolytic reaction, it is suggested that the phosphate binds first to the α/β domain leading to a structural rearrangement and a partial closing of the cleft. Subsequent binding of the pyrimidine 2′-deoxyriboside causes the cleft to close, excluding water and leading to the phosphorolytic event, before reopening with the pyrimidine bound to the α-helical domain. Release of the pyrimidine could then be followed either by binding of another pyrimidine (leading to transferase activity) or by release of the 2-deoxyribose-1-phosphate [5]. The distance between the phosphate-binding site and the Thy/dThd-binding site is estimated to be ∼10 Å in the open protein (Fig. 1).

Interest in TPase has markedly increased in the light of several recent findings (reviewed in [6–8]). Firstly, TPase expression is reported to be inducible by interferon and other cytokines [7]. Secondly, platelet-derived endothelial cell growth factor has been shown to be identical to human TPase [9–12]. Thus, TPase possesses angiogenic and endothelial cell chemotactic activities. Thirdly, gliostatin, which causes inhibition of glial cell growth and subsequent stimulation of neuronal outgrowth, has also been shown to be identical to TPase [7]. The mode of action of TPase as a growth factor is presently not fully understood, although it has been suggested that the responsible agent is 2-deoxyribose, derived from the 2-deoxyribose-1-phosphate released in the TPase reaction [8].

Few inhibitors of TPase have been reported, most of them being uracil derivatives such as 6-amino-5-bromouracil

*Corresponding author. Fax: (32)-16-33 73 40.
E-mail: jan.balzarini@rega.kuleuven.ac.be

0014-5793/00/$20.00
PII: S0014-5793(00)02101-3

(6A5BU) (Fig. 2) and 6-aminothymine, albeit with IC_{50} values in the sub-micromolar range [13]. Very recently, 5-chloro-6-[1-(2-iminopyrrolidin)methyl]uracil has been described as a novel powerful inhibitor with an IC_{50} value of 33 nM [14]. In our initial attempt to identify new inhibitors of TPase, molecular modeling was performed based on the medium resolution of the *E. coli* TPase structure. Synthesis and resolution of several structures derived from our modeling led to the identification of 7-deazaxanthine as the first purine derivative able to inhibit TPase [15]. In a further step, and again based on the available *E. coli* coordinates, several series of compounds were designed and synthesised, in an attempt to interact simultaneously with both the phosphate and the pyrimidine-binding site. From these investigations, the most potent inhibitors proved to be the 1-(8-phosphonooctyl) derivatives of 6A5BU and 7-deazaxanthine [16]. Here, we report a detailed kinetic analysis of the interaction of these novel compounds with bacterial TPase and demonstrate that they indeed interfere with the two different sites of the enzyme. To the best of our knowledge they represent the first example of multisubstrate analogue inhibitors of TPase.

2. Materials and methods

2.1. Molecular modeling procedures

The full-atom model of *E. coli* TPase was kindly provided by Dr. S. Ealick (Cornell University, NY, USA) and the inhibitors 6A5BU and 6-aminothymine were modeled into the active site based on the position of thymine (Thy) inferred by Walter et al. [15]. The position of the phosphate-binding site was inferred from the position of a bound sulphate ion in the crystal structure (a result of the crystallisation conditions). The rationale behind the design of 7-deazaxanthine has been described [15]. Phosphonates were considered to be good analogues for the phosphate ion, leaving the choice of linker to be made. Several types of linkers were considered, as described in [16], with their lengths and positions of functional groups/unsaturated bonds being determined by manual model building between the base and phosphate positions. The goal of the model building was to identify ways of reducing the flexibility of the linker region (to reduce the entropic penalty of binding the groups at either end of the linker) and to attempt to form additional favourable linker–protein interactions (particularly with residue His85).

2.2. Compounds

The synthesis of 7-deazaxanthine was performed according to West et al. [17]. Thymidine (dThd) and Thy, and *E. coli* TPase (1030 U/ml) were obtained from Sigma (St. Louis, MO, USA). 6A5BU was synthesised according to Schroeder [18]. The synthesis of the 1-(8-phosphonooctyl) derivative of 6A5BU (TP-64) and 1-(8-phosphonooctyl)-7-deazaxanthine (TP-65) has been recently described [16]. The structural formulae are depicted in Fig. 2.

2.3. TPase enzyme assays

The phosphorolysis of dThd by *E. coli* TPase was measured by high performance liquid chromatography (HPLC) analysis. The incubation mixture (500 μl) contained 10 mM Tris–HCl (pH 7.6), 1 mM EDTA, 2 mM potassium phosphate (unless otherwise stated in the kinetic experiments), 150 mM NaCl and 100 μM of dThd in the presence or absence of 100 μM 2-deoxyribose-α-1-phosphate and 0.025 U TPase. Incubations were performed at room temperature. At different time points (i.e. 0, 20, 40 and 60 min), 100 μl fractions were withdrawn, transferred to an Eppendorf tube thermo block, and boiled at 95°C for 5 min. Thereafter, the samples were rapidly cooled on ice, and dThd was separated from Thy and quantified in the samples on a reverse phase RP-8 column (Merck, Darmstadt, Germany) by HPLC analysis. The separation of Thy and dThd was performed by a linear gradient from 5% buffer B (10 mM potassium phosphate buffer pH 5.5+80% methanol) to 20% buffer B. After injection of the samples, buffer B was given for 10 min before the start of the gradient (5 min from 5 to 20% buffer B). The retention times of Thy and dThd were 4.2 and 8.5 min, respectively. UV-based detection of Thy and dThd was performed at 267 nm.

In the assays where the inhibitory effect of the compounds was evaluated, a variety of inhibitor concentrations, including 1 mM, 100, 10 and 0 μM (control) were added to the reaction mixture (500 μl) containing 100 μM of dThd. Aliquots of 100 μl were withdrawn from the reaction mixture at several time points, as described above, heated at 95°C to inactivate the enzyme, and analysed on HPLC.

To discriminate between reversible and irreversible inhibition of TPase by the inhibitors, 100 μM 7-deazaxanthine and TP-65 were exposed to different TPase concentrations (0.005, 0.004, 0.003, 0.0025, 0.0020, 0.001 and 0.0005 U) in a 100 μl reaction mixture and incubated for 30 min at room temperature. Conversion of dThd to Thy was followed as described above.

In the kinetic assays, where the inhibitory effect of the test compounds was evaluated against TPase at varying inorganic phosphate (Pi) concentrations, the compounds were tested at concentrations ranging from 0.5 to 200 μM, in the presence of 2, 5, 10, 25, 50 and 100 mM Pi. The (saturating) dThd concentration was kept fixed at 1000 μM. The reaction mixture (100 μl) was then incubated at room temperature for 20 min with 0.005 U TPase, after which the tubes were heated at 95°C before cooling and analysis by HPLC for dThd conversion to Thy.

In the kinetic assays, where the inhibitory effect of the test compounds was evaluated against TPase at varying Thy or dThd concentrations, the compounds were tested at concentrations ranging from 2 to 200 μM or 0.5 to 200 μM, in the presence of 175, 250, 375, 500, 750 and 1000 μM Thy or 125, 250, 500, 750 and 1000 μM dThd, respectively. The Pi concentration was kept constant at 25 mM. The analysis of the Thy-to-dThd conversion was performed as described above.

The data from the kinetic experiments were fitted to standard enzyme inhibition models (i.e. competitive, non-competitive, mixed) using Sigma Plot Version 4.0 and are summarised in Table 1. The best fit was judged from the non-reduced χ^2 values (sum of squared residuals) given by the program and the percentage of errors in the fitted parameters.

3. Results

To enable discrimination between reversible and irreversible

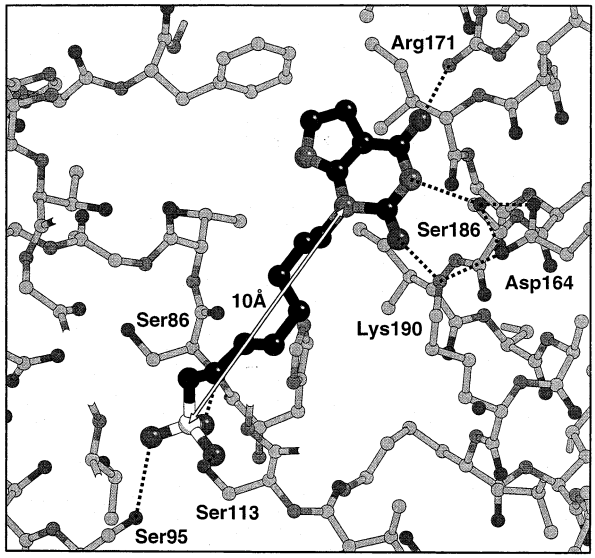

Fig. 1. A ball-and-stick diagram of *E. coli* dThd kinase [3] with one of the multisubstrate inhibitors (TP-65) modelled into the active site. Hydrogen bonding interactions between the 7-deazaxanthyl moiety and the enzyme and between the phosphonyl moiety and the enzyme are shown by dotted lines. The distance between the binding sites in the open form of the enzyme is indicated. Figure was drawn using BobScript [19].

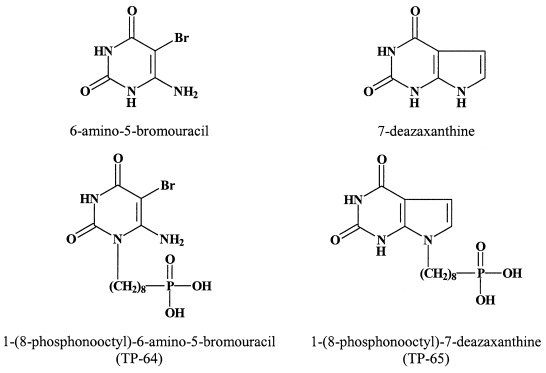

Fig. 2. Structural formulae of the substrate analogue inhibitors 6A5BU and 7-deazaxanthine and the multisubstrate analogue inhibitors TP-64 and TP-65.

inhibition of TPase by 7-deazaxanthine and its phosphonoalkyl derivative (TP-65), a fixed concentration of 100 μM of the inhibitors was exposed to duplo-dilutions of TPase. Should the compounds act as irreversible inhibitors of TPase, equimolar concentrations of enzyme and inhibitor would completely annihilate enzymatic activity and, likewise, lower enzyme concentrations will be fully inactivated by the compounds. Consequently, if enzyme concentration was graphically plotted in the absissa and enzyme velocity in the ordinate, the enzyme activity curve (in the presence of the inhibitor) should follow a parallel line with the enzyme activity curve of control (without inhibitor). If, however, the enzyme inhibition is reversible, both the enzyme activity curve (in the presence of the inhibitor) and the enzyme activity curve of control (without inhibitor) would converge to the intersec-

Table 1
Kinetic parameters for different inhibitors and substrates (phosphate, dThd and Thy) according to different enzyme inhibition models

Substrate and inhibitor	K_m (±S.E.M.) (μM)	K_i (±S.E.M.) (μM)	K_{ii} (±S.E.M.) (μM)	V_{max} (±S.E.M.) (nmol/h×10^{-3})	Non-reduced χ^2 value
Thymidine and 6-amino-5-bromouracil:					
Competitive	9437 ± 1742	0.80 ± 0.12	–	1718 ± 171	183.2
Mixed	11065 ± 2018	1.4 ± 0.45	1.9 ± 0.19	1875 ± 195	159.1
Non-competitive	11498 ± 1647	1.6 ± 0.16	–	1914 ± 162	159.6
Thymidine and 7-deazaxanthine:					
Competitive	5199 ± 800	7.7 ± 1.2	–	1231 ± 82.4	162.3
Mixed	6353 ± 1017	13 ± 3.6	31 ± 14	1357 ± 104	140.8
Non-competitive	7365 ± 866	19 ± 2.0	–	1454 ± 90	147.2
Thymidine and TP-64:					
Competitive	3865 ± 337	142 ± 11	–	731 ± 31.3	32.1
Mixed	4209 ± 441	179 ± 33	850 ± 617	763 ± 41	30.2
Non-competitive	5084 ± 434	297 ± 19	–	842 ± 40.6	35.2
Thymidine and TP-65:					
Competitive	4500 ± 547	54 ± 4.7	–	793 ± 50.6	41.1
Mixed	4499 ± 563	53 ± 4.9	$5.7\times10^6 \pm 5.1\times10^8$	793 ± 52.1	41.1
Non-competitive	6160 ± 857	105 ± 8.5	–	9.43 ± 78.9	51.1
Thymine and 6-amino-5-bromouracil:					
Competitive	1253 ± 206	2.3 ± 0.35	–	642 ± 32.1	93.8
Mixed	1395 ± 250	3.06 ± 0.82	34.7 ± 29.1	665 ± 38.9	90.0
Non-competitive	2084 ± 316	8.7 ± 0.91	–	761 ± 48.5	114.0
Thymine and 7-deazaxanthine:					
Competitive	1123 ± 94.9	16 ± 1.37	–	612 ± 14.8	47.0
Mixed	1234 ± 107	21 ± 3.0	254 ± 120	630 ± 16.6	41.8
Non-competitive	1745 ± 175	62 ± 4.9	–	702 ± 27.4	73.2
Thymine and TP-64:					
Competitive	2100 ± 151	312 ± 39.7	–	225 ± 6.28	17.0
Mixed	2327 ± 214	476 ± 160	1419 ± 887	235 ± 9.1	15.9
Non-competitive	2541 ± 156	792 ± 84.3	–	244 ± 6.9	16.6
Thymine and TP-65:					
Competitive	2074 ± 248	93 ± 10.4	–	220 ± 10.3	22.5
Mixed	2335 ± 323	125 ± 30.0	700 ± 477	231 ± 13.4	21.3
Non-competitive	2975 ± 317	251 ± 21.4	–	256 ± 13.3	24.2
Phosphate and 6-amino-5-bromouracil:					
Competitive	2160 ± 1660	0.035 ± 0.025	–	503 ± 46	206
Mixed	4390 ± 380	0.74 ± 0.18	0.78 ± 0.04	588 ± 9.2	32.6
Non-competitive	4440 ± 300	0.77 ± 0.03	–	588 ± 8.2	32.6
Phosphate and 7-deazaxanthine					
Competitive	653 ± 1068	1.0 ± 1.6	–	619 ± 45	280
Mixed	3524 ± 424	49 ± 29	27 ± 1.9	797 ± 16	60.3
Non-competitive	3225 ± 300	29 ± 1.6	–	789 ± 14	61.8
Phosphate and TP-64					
Competitive	2749 ± 290	35 ± 4.8	–	948 ± 11	68
Mixed	2796 ± 338	36 ± 6.0	$20\times10^3 \pm 69\times10^3$	951 ± 15	68
Non-competitive	6974 ± 1060	565 ± 138	–	1050 ± 43	191
Phosphate and TP-65					
Competitive	3666 ± 502	29 ± 5.9	–	1667 ± 29	180
Mixed	4327 ± 563	40 ± 9.6	887 ± 390	1727 ± 37	159
Non-competitive	7222 ± 820	300 ± 57	–	1840 ± 55	254

K_m is the Michaelis–Menten constant of the enzyme, K_i is the dissociation constant of the enzyme, K_{ii} is the dissociation constant of the enzyme–substrate complex, V_{max} is the maximum velocity of the enzyme reaction.

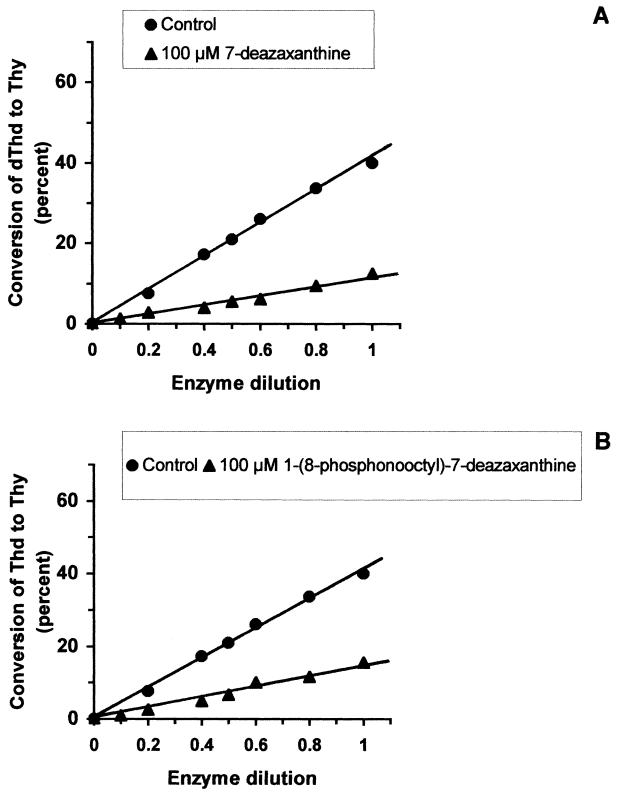

Fig. 3. Reversible inhibition of TPase by 7-deazaxanthine (A) and TP-65 (B). A concentration of 100 μM inhibitor was used in the enzyme reaction and different concentrations of the enzyme. dThd was used as the natural substrate.

tion (zero) point of the absissa/ordinate graph. As evident from Fig. 3, the latter situation proved to be the case. Thus, both compounds (7-deazaxanthine (Fig. 3A) and TP-65 (Fig. 3B)) behaved as reversible inhibitors of the enzyme.

The inhibitory activities (potencies) of the test compounds against TPase have been previously evaluated in the presence of 100 μM dThd [16]. The kinetic parameters, including the nature of inhibition and the K_i values of the inhibitors, have now been determined in the presence of varying concentrations of inhibitor and dThd (Table 1). 6A5BU emerged as the most potent inhibitor of TPase with a K_i/K_m value around 0.0001, and inhibited the enzyme reaction in a mixed or non-competitive fashion with respect to dThd as the variable substrate. Also 7-deazaxanthine showed a mixed mode of inhibition that was closer to non-competitive than competitive as judged from the non-reduced χ^2 (sum of the squared residuals) values. In contrast, TP-64 and TP-65 were less inhibitory than 6A5BU and 7-deazaxanthine, but inhibited the enzyme reaction in a mixed or competitive fashion with respect to dThd (Table 1).

We then examined the nature of the kinetic interaction of 6A5BU, 7-deazaxanthine, TP-64 and TP-65 with TPase with respect to varying concentrations of Thy. The (saturating) Pi concentrations were kept constant during the enzyme reactions. As shown in Table 1, a comparison of the non-reduced χ^2 values for the different kinetic models shows that 6A5BU and 7-deazaxanthine invariably inhibited the enzyme in a mixed fashion with respect to Thy as the varying substrate. However, a strong tendency to competitive inhibition was observed (non-reduced χ^2 values for mixed inhibition were much closer to the non-reduced χ^2 values for competitive inhibition than for non-competitive inhibition). Also, the phosphonoalkyl derivatives of 6A5BU and 7-deazaxanthine showed a mixed mode of inhibition, but based on the non-reduced χ^2 values, it was difficult to delineate a predominant tendency to either a competitive or a non-competitive mode of action. The K_i values (for the best fit) were around 3.1, 21, 476 and 125 μM for 6A5BU, 7-deazaxanthine, TP-64, and TP-65, respectively, resulting in K_i/K_m values that ranged from 0.002 to 0.2. The order and degree of potency of the different inhibitors was very similar to that obtained with dThd as the competing substrate (Table 1).

TP-64 and TP-65 were originally designed to enable a concomitant interaction of these types of compounds with both the Thy/dThd substrate-binding site and the phosphate-binding site of the enzyme. To assess whether the phosphonoalkyl derivatives of 6A5BU and 7-deazaxanthine are really interacting at the phosphate-binding site, the phosphonate inhibitors as well as their free base analogues (6A5BU and 7-deazaxanthine) were exposed to the enzyme in the presence of various Pi concentrations at a fixed (high) concentration of dThd. The mixed or purely competitive model was strongly preferred over the non-competitive model for both phosphonoalkyl derivatives TP-64 and TP-65 (K_i/K_m values of $\sim$0.01). In contrast, the free base inhibitors 6A5BU and 7-deazaxanthine behaved as non-competitive inhibitors of the enzyme with respect to Pi (K_i/K_m values: 0.0002 and 0.01, respectively). Thus, whereas the free base analogues can bind independently of, and concomitantly with, Pi to the enzyme, binding of the phosphonoalkyl derivatives of these base analogues proved mutually exclusive with Pi or changed the substrate affinities of the enzyme upon binding with the inhibitor.

Taken together, the kinetic data presented in Table 1 strongly suggest that the novel phosphonoalkyl derivatives of 6A5BU and 7-deazaxanthine may interfere with both the phosphate- and the Thy/dThd-binding site of the enzyme and, thus, may be considered as multisubstrate analogue inhibitors of TPase.

4. Discussion

Almost all known inhibitors of TPase can be described as uracil derivatives interacting at the Thy/dThd-binding site. Based on the X-ray coordinates published for the *E. coli* enzyme, we have recently designed and synthesised several series of compounds that should interact both at the Thy/dThd and the phosphate-binding site, and thus that should behave as multisubstrate analogue inhibitors of TPase. From the synthesised compounds, the most potent inhibitors of TPase turned out to be TP-64 and TP-65 [16]. The kinetics of these compounds and of their corresponding free bases, 6A5BU and 7-deazaxanthine, against bacterial TPase in the presence of different substrates have now been examined. The results obtained clearly indicate that the phosphonoalkyl derivatives TP-64 and TP-65 inhibited TPase in a competitive or mixed fashion with respect to both dThd and Pi as the natural substrates, while 6A5BU and 7-deazaxanthine behaved as non-competitive inhibitors of the enzyme with respect to phosphate. These data convincingly argue for an interference of TP-64 and TP-65 at both substrate (dThd and Pi)-binding

sites of the enzyme, as originally designed. It could have been expected that such multisubstrate analogue inhibitors would be more potent inhibitors than their corresponding free bases. However, the linker region connecting the two substrate analogues in these molecules loses a lot of conformational freedom on binding to TPase and the resulting loss of entropy will affect the potency of these compounds adversely. Less flexible linkers would thus be advantageous and work is already underway to develop these new molecules.

The observed kinetic nature of the interaction of the compounds with the enzyme depended on whether Thy or dThd was used as the natural substrate. The most striking difference was the (expected) trend to competitive inhibition of 6A5BU when Thy was used, but (unexpected) trend to non-competitive inhibition when dThd was the variable substrate. We have currently no explanation for this phenomenon. However, the conformation of the enzyme active site is likely to be somewhat different depending on whether Thy or dThd are bound. It is assumed that during catalysis a domain movement (closing) must occur to produce an active conformation allowing the phosphate to contact the anomeric position of dThd followed by a re-opening that allows the Thy to diffuse from the enzyme. In this respect, a very recent study shows discrepancies between the binding mode of dThd, based on docking programmes (Autodock), and the binding mode of minimised dThd based on bound Thy in the *E. coli* TPase coordinates [6]. The authors of this study concluded that the *E. coli* X-ray structure resembles more the enzyme-diffusing Thy conformation than the enzyme-binding dThd conformation. Thus, the binding modes of the free base analogues, and even more of the corresponding phosphonoalkyl derivatives, may be different under different experimental conditions (varying concentrations of Thy and dThd), resulting in a different mode of interaction with the enzyme. It is imperative to obtain and analyse enzyme crystals complexed with the novel multifunctional inhibitors to understand these differences in the interaction of the enzyme with the inhibitors at the molecular level.

The described phosphonoalkyl derivatives were designed based on the open conformation of the enzyme, as shown in Fig. 1, and the kinetic data presented here confirmed that they are indeed able to interfere both at the dThd and at the phosphate-binding sites. Only cocrystallisation of TPase with these phosphonoalkyl derivatives should provide formal proof that these compounds are indeed able to (reversibly) freeze the conformation of TPase in an open, inactive form.

Since multisubstrate analogue inhibitors have yielded extremely potent inhibitors of other enzymes, including purine nucleoside phosphorylases, we expect that our lead compounds may form the basis for further improvement of their inhibitory activity against TPase, and may result in the very first potent multisubstrate inhibitor analogues for TPase. The availability of such potent and specific TPase inhibitors may allow investigation of their role in the inhibition of angiogenesis and cancer.

Acknowledgements: We thank Mrs. Ria Van Berwaer for excellent technical assistance and Mrs. Christiane Callebaut for fine editorial help. This research was supported by grants from the European Commission (PL96-2161), the Geconcerteerde Onderzoeksacties, Vlaanderen (GOA-12/00), the Belgian Federation against Cancer and from the Spanish CICYT (SAF 2000-0153-C02-01).

References

[1] Ilstzsch, M.H., el Kouni, M.H. and Cha, S. (1985) Biochemistry 24, 6799–6807.
[2] Walter, M.R., Cook, W.J., Cole, L.B., Short, S.A., Koszalka, G.W., Krenitsky, T.A. and Ealick, S.E. (1990) J. Biol. Chem. 265, 14016–14022.
[3] Pugmire, M.J. and Ealick, S.E. (1998) Structure 6, 1467.
[4] Pugmire, M.J., Cook, W.J., Jasanoff, A., Walter, M.R. and Ealick, S.E. (1998) J. Mol. Biol. 281, 285–299.
[5] Schwartz, M. (1971) Eur. J. Biochem. 21, 191–198.
[6] Cole, C., Marks, D.S., Jaffar, M., Stratford, I.J., Douglas, K.T. and Freeman, S. (1999) Anti-Cancer Drug Des. 14, 411–420.
[7] Griffiths, L. and Stratford, I.J. (1997) Br. J. Cancer 76, 689.
[8] Moghaddam, A., Choudhuri, R. and Bicknell, R. (1997) in: Tumor Angiogenesis (Bichnell, R., Lewis, C.E. and Ferrara, N., Eds.). Oxford University Press, Oxford.
[9] Miyazono, K., Okabe, T., Urabe, A., Takaku, F. and Heldin, C.H. (1987) J. Biol. Chem. 262, 4098–40103.
[10] Moghaddam, A., Zhang, H.T., Fan, T.P., Hu, D., Lees, V.C. and Turley, H. et al. (1995) Proc. Natl. Acad. Sci. USA 92, 998–1002.
[11] Furukawa, T., Yoshimura, A., Sumizawa, T., Haraguchi, M. and Akiyama, S.I. (1992) Nature 356, 669.
[12] Finnis, C., Dodsworth, N., Pollit, C.E., Carr, G. and Sleep, D. (1993) Eur. J. Biochem. 212, 201–210.
[13] Langen, P., Etzold, G., Bärwolff, D. and Preussel, B. (1967) Biochem. Pharmacol. 16, 1833–1837.
[14] Matsushita, S., Nitanda, T., Furukawa, T., Sumizawa, T., Tani, A., Nishimoto, K., Akiba, S., Miyadera, K., Fukushima, M., Yamada, Y., Yoshida, H., Kanzaki, T. and Akiyama, S.-i. (1999) Cancer Res. 39, 1911–1916.
[15] Balzarini, J., Esteban-Gamboa, A., Esnouf, R., Liekens, S., Neyts, J., De Clercq, E., Camarasa, M.J. and Pérez-Pérez, M.J. (1998) FEBS Lett. 438, 91–95.
[16] Esteban-Gamboa, A., Balzarini, J., Esnouf, R., De Clercq, E., Camarasa, M.-J. and Pérez-Pérez, M.-J. (2000) J. Med. Chem. 43, 973–983.
[17] West, R.A., Ledig, K. and Hitchings, G.H. (1959) Br. Patent 812.366, Chem. Abst. 1960, 54, 5921.
[18] Schroeder, E.F. (1956) US Patent 2.731.465, Chem. Abst. 1957, 51, 1257.
[19] Esnouf, R.M. (1999) Acta Cryst. D 55, 938–940.