7-Deazaxanthine, a novel prototype inhibitor of thymidine phosphorylase

Jan Balzarini[a,*], Antonio Esteban Gamboa[b], Robert Esnouf[a], Sandra Liekens[a], Johan Neyts[a], Erik De Clercq[a], Maria-José Camarasa[b], Maria-Jesus Pérez-Pérez[b]

[a] *Rega Institute for Medical Research, K.U. Leuven, Minderbroedersstraat 10, B-3000 Louvain, Belgium*
[b] *Instituto de Química Médica, CSIC, 28006 Madrid, Spain*

Received 20 August 1998

Abstract 7-Deazaxanthine (7DX) was identified as a novel inhibitor of thymidine (dThd) phosphorylase (TPase). It inhibited the TPase reaction in a concentration-dependent manner. At 1 mM, it almost completely prevented the TPase-catalysed hydrolysis of dThd to thymine. The 50% inhibitory concentration (IC_{50}) of 7DX was 40 μM in the presence of 100 μM of the natural substrate dThd. 7DX is also endowed with a marked inhibitory effect on angiogenesis. It significantly prevents neovascularisation in the chicken chorioallantoic membrane during development. 7DX is the first purine derivative shown to be a potent inhibitor of purified TPase and angiogenesis.

© 1998 Federation of European Biochemical Societies.

Key words: Thymidine phosphorylase; Angiogenesis; 7-Deazaxanthine; 6-Amino-5-bromouracil; TPase inhibitor

1. Introduction

There exist at least two different pyrimidine nucleoside phosphorylases that catalyse the conversion of pyrimidine (deoxy)nucleosides and structurally related analogues in mammalian cells: thymidine phosphorylase (TPase; EC 2.4.2.4) and uridine phosphorylase (UPase, EC 2.4.2.3). TPase is highly specific for the deoxyribosides of thymine and uracil analogues. A variety of nucleoside analogues that are endowed with antiviral activity (i.e. (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU), 5-trifluoromethyl-2′-deoxyuridine (TFT), 5-iodo-2′-deoxyuridine (IdU)) and antitumour activity (i.e. 5-fluoro-2′-deoxyuridine (FdU)) are good substrates for TPase [1]. Consequently, the presence of TPase activity in blood (platelets) and in cells may cause a progressive inactivation of these antiviral and anticancer agents (due to the catabolism of the drugs), thereby preventing or diminishing the anabolic conversion of the drugs to their biologically active metabolites.

It has recently been shown that platelet-derived endothelial cell growth factor (PD-ECGF) is endowed with TPase activity [2–4], and that this enzymatic activity is crucial for the angiogenic effect [5–7]. Histological analysis of a variety of human tumours including bladder [8], breast [9], oesophageal [10], and colorectal cancers [11], have shown elevated PD-ECGF/TPase levels. These levels correlated well with microvessel density in breast [12], colon [11] oesophageal [10] and renal cancers [13], providing evidence that PD-ECGF/TPase contributes to the tumour vasculature [14]. Thus, PD-ECGF is known to stimulate endothelial cell migration in vitro and angiogenesis in vivo.

There is an urgent need for developing potent and selective inhibitors of TPase, because they may potentiate the antiviral activity of certain nucleoside analogues and/or they may be endowed with anti-tumour (i.e. anti-angiogenic) activity. To date, very few inhibitors of TPase have been described, 6-aminothymine (6AT) and 6-amino-5-bromouracil (6A5BU) ranking among the most potent [15].

In this report, we describe the first purine derivative (7-deazaxanthine, 7DX) that shows inhibitory activity against *Escherichia coli* TPase and that could be considered a novel lead compound in the development of TPase inhibitors. Furthermore, this compound was found to inhibit the angiogenesis in the chicken chorioallantoic membrane.

2. Materials and methods

2.1. Compound

The synthesis of 7-deazaxanthine was performed according to West et al. [16]. Thymidine and thymine, and *E. coli* thymidine phosphorylase (TPase) (1030 units/ml) were obtained from Sigma (St. Louis, MO). 6-Amino-5-bromouracil (6A5BU) was synthesised according to E.F. Schroeder [17]. The structural formulae of the compounds are depicted in Fig. 1.

2.2. TPase enzyme assay

The phosphorolysis of thymidine (dThd) by *E. coli* TPase was measured by HPLC analysis. 1 ml of the incubation mixture contained 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 2 mM potassium phosphate, 150 mM NaCl, and 100 μM of thymidine in the presence or absence of 100 μM 2-deoxyribose-α-1-phosphate (α-dR-1-P) and 0.05 units of TPase. Incubations were performed at 37°C. At different time points (i.e. 0, 10, 20, 40 and 60 min), 200-μl fractions were taken, brought into 1.5-ml Eppendorf tubes and rapidly cooled on ice. When all samples were collected, they were transferred to an Eppendorf tube thermo block and boiled at 95°C for 5 min. The dThd was separated from Thy and quantified in the samples by HPLC analysis.

In the assays where the inhibitory effects of 6A5BU and 7DX were evaluated, a variety of inhibitor concentrations, including 1 mM, 250 μM, 100 μM, 25 μM, 10 μM and 0 μM (control), were added to the reaction mixture that contained 100 μM of dThd. Aliquots of 200 μl were withdrawn from the reaction mixture at several time points, as described above, and analysed on HPLC.

2.3. Chorioallantoic membrane assay in fertilised chicken eggs

The chorioallantoic membrane (CAM) angiogenesis assay was performed as originally described by Maragoudakis et al. [18]. Fertilised eggs were incubated for 4 days at 37°C. Then, a window of approximately 1 cm^2 was opened on the egg shell exposing the CAM, and covered with cellophane tape. The eggs were further incubated at 37°C for an additional 5 days (day 9) at which time the test compounds (6A5BU and 7DX) were administered. The test compounds were placed on sterile plastic discs (10 mm diameter) at 1 μmol, 250 nmol and 100 nmol in DMSO and allowed to dry under sterile conditions. The control discs (containing DMSO) were placed on the CAM 1 cm away from the disc containing the test material. A solution of cortisone acetate (100 μg/disc, Sigma, St. Louis, MO) was incorporated in all discs to prevent an inflammatory response. The loaded and dried discs were inverted and placed on the CAM, the

*Corresponding author. Fax: (32) (16) 33.73.40.

0014-5793/98/$19.00 © 1998 Federation of European Biochemical Societies. All rights reserved.
PII: S0014-5793(98)01271-X

windows were covered with cellophane tape again, and the eggs further incubated at 37°C until day 11.

The eggs were then flooded with 10% buffered formalin (Janssen Chimica, Geel, Belgium), the plastic discs were carefully removed and the eggs were kept at room temperature for at least 4 h. A large area around the discs was cut off and placed on a glass slide, and the vascular density index (expressed as the number of blood vessels) was measured by the method of Harris-Hooker et al. [19]. This method allows an objective evaluation of microvessel formation, taking into account the small newly formed microvessels.

3. Results

3.1. Design of 7DX

Since the three-dimensional structure of TPase from *E. coli* has been determined at 2.8 Å resolution [20], rational modelling and design of novel inhibitors of the enzyme has become a feasible goal. The coordinates for the full-atom model of *E. coli* TPase obtained crystallographically were kindly provided by Dr. S. Ealick (Cornell University, NY). The well known inhibitors 6A5BU and 6AT were modelled into the active site based on the position of thymine inferred by Walter et al. [20]. Novel potential inhibitors of TPase were designed which preserved the structural features of these inhibitors, and their interactions with TPase, as much as possible. The compound 7DX was one of these designs where the addition of the second ring was intended to create extra stabilising interactions by filling the apparent space near the ring of residue Phe^{210}.

3.2. Inhibitory effect of 7DX against purified TPase

In a first set of experiments, 100 μM 7DX was added to a reaction mixture containing equimolar amounts of dThd, and the inhibitory effect of 7DX on the TPase-catalysed conversion of dThd to Thy+α-dR-1-P was examined. The well-known TPase inhibitor 6A5BU was added for comparative reasons in parallel experiments under similar experimental conditions. The time-dependent dThd degradation in the presence of 100 μM 7DX or 6A5BU or in the absence of the inhibitors is presented in Fig. 2. 7DX was found to be a

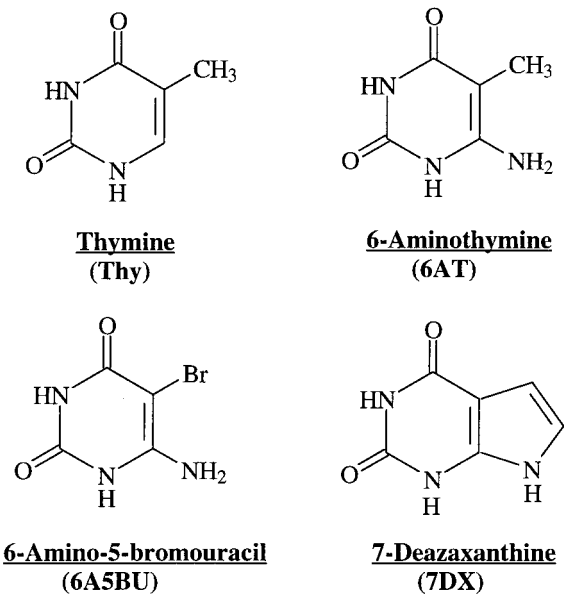

Fig. 1. Structural formulae of thymine (Thy), 6-aminothymine (6AT), 6-amino-5-bromouracil (6A5BU) and 7-deazaxanthine (7DX).

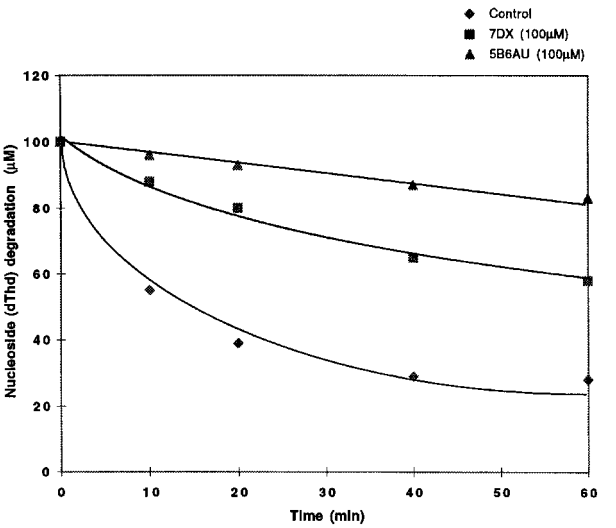

Fig. 2. Inhibition of TPase-mediated thymidine degradation (phosphorolysis) by 100 μM 6A5BU and 100 μM 7DX as a function of incubation time. The initial thymidine concentration in the reaction mixture was 100 μM.

potent inhibitor of the TPase reaction. After 10 min, when approximately 50% of dThd was converted to Thy in the control reaction, dThd conversion was inhibited by 74% in the presence of 7DX. At 20, 40 and 60 min, when dThd conversion to Thy reached its equilibrium, the inhibitory potential of 7DX progressively decreased from 68% (20 min) to 52% (40 min), eventually reaching 41% at 60 min. When the potent TPase inhibitor 6A5BU was evaluated under similar experimental conditions, the hydrolysis reaction was inhibited by 91%, 88%, 82% and 76% after 10, 20, 40 and 60 min, respectively, of incubation of the enzyme with 100 μM dThd and 100 μM 6A5BU. Virtually identical values were obtained when α-dR-1-P was added to the reaction mixture (data not shown). It should also be mentioned that under our experimental conditions, no significant conversion of 6A5BU and 7DX to their corresponding nucleoside derivatives was observed on the HPLC chromatograms.

3.3. Concentration-inhibitory response of 7DX against E. coli TPase

Different concentrations of 7DX and 6A5BU (i.e. 1 mM, 250 μM, 100 μM, 25 μM and 10 μM) were exposed to the TPase reaction using 100 μM dThd as the natural substrate. 7DX prevented dThd hydrolysis by 96% at 1 mM and by 89% at 250 μM after 10 min (Table 1); at the lower concentrations (i.e. 25 and 10 μM), 7DX was still 43% and 25% inhibitory (Table 1). At 60 min, when the control reaction had virtually reached an equilibrium (completion), inhibition was still pronounced at the high 7DX concentrations (91% and 73% at 1 mM and 250 μM, respectively) but less pronounced at the lower concentrations (7% and 4% at 25 μM and 10 μM, respectively) (Table 1). The 6A5BU derivative proved virtually equipotent an inhibitor of the TPase reaction as 7DX at 250 μM, but superior to 7DX at lower concentrations (Table 1). In both cases, the inhibitory effect of 7DX and 6A5BU was more pronounced when determined within 10 min of the enzyme reaction than within 60 min of the enzyme reaction (Table 1).

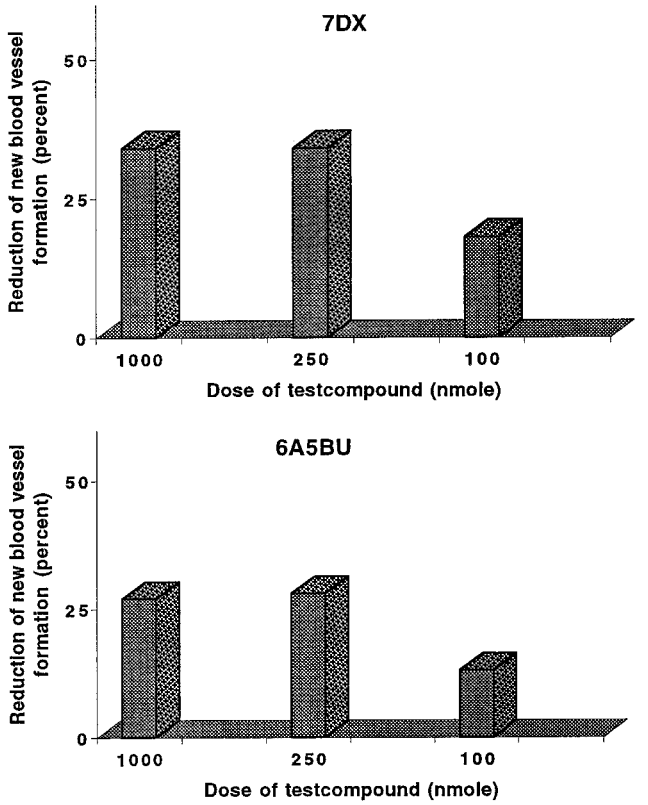

Fig. 3. Inhibition of new blood vessel formation in the chicken egg CAM test by 1000, 250 or 100 nmol of 7DX and 6A5BU.

3.4. Inhibitory effect of 7DX on neovascularisation in the CAM assay

The TPase inhibitors 7DX and 6A5BU were exposed to the CAM of fertilised chicken eggs at 1 μmol, 250 nmol and 100 nmol. At least 10 eggs were scored for each inhibitor concentration in three independent experiments. Both 7DX and 6A5BU markedly prevented blood vessel formation in the CAM test during a 2-day exposure time period (Figs. 3 and 4): when exposed to 7DX at 1 μmol, 250 nmol or 100 nmol, the eggs developed 34%, 34% or 18% less vessels than control eggs. The TPase inhibitor 6A5BU afforded a reduction in new vessel formation of 27%, 28% and 13% at 1 μmol, 250 nmol and 100 nmol, respectively (Fig. 3). The P values for the 1 μmol and 250 nmol compound doses were < 0.05 when compared to control, whereas there was no statistical difference between the inhibitory values of 6A5BU and 7DX at equimolar concentrations. Thus, 7DX and 6A5BU proved equipotent as inhibitors of angiogenesis in the CAM assay. Moreover, the reference anti-angiogenic fumagillin analogue TNP-470, which is subject to phase I, II and III clinical trials for a variety of solid tumours including Kaposi's sarcoma, afforded at a maximum tolerable dose of 100 nmol 21% inhibition of neovascularisation, under the same experimental condition at which the subtoxic dose of 100 nmol 7DX afforded 34% inhibition (data not shown).

4. Discussion

7-DX should be considered a new lead compound with pronounced inhibitory activity against thymidine phosphorylase and angiogenesis. To the best of our knowledge 7DX is the first purine derivative that has been recognised as an efficient inhibitor of a pyrimidine nucleoside phosphorylase (i.e. TPase) and should be maintained in the future design of new TPase inhibitors. Indeed, the CO-NH-CO functional entity in the pyrimidine ring is also present in 7-DX. Moreover, the 5-methyl part of thymine corresponds to the ethenyl moiety of the pyrrole ring of 7DX, whilst the 6-amino group of 6AT and 6A5BU corresponds to the NH of 7DX. The fact that 7DX contains a second (pyrrole) ring entity in the molecule may open interesting perspectives in the design of more potent purine-based TPase inhibitors. The inhibitory activity noted for 7DX against TPase proved to be at the same order of magnitude as 6A5BU, and supports our modelling studies that revealed that 7DX mimics thymine and 6AT in the TPase enzyme active site.

An important observation is that the TPase inhibition by 7DX and 6A5BU measured in the enzymatic assays closely correlates with the anti-angiogenic effect of these compounds

Table 1
Inhibitory activity of 7DX and 6A5BU against thymidine phosphorylase as a function of compound concentration and incubation time

Inhibitor concentration (μM)	10 min incubation		60 min incubation	
	% of dThd remaining[a]	% Inhibition of TPase[b]	% of dThd remaining[a]	% Inhibition of TPase[b]
7DX				
1000	98	96	93	91
250	95	89	80	73
100	88	74	58	41
25	74	43	32	7
10	66	25	30	4
0	55	0	28	0
6A5BU				
1000	100	100	100	100
250	96	91	83	76
100	94	87	80	71
25	89	76	59	44
10	82	60	42	20
0	55	0	28	0

[a]Percentage of thymidine remaining in the reaction mixture, as assessed by the HPLC chromatogram used to monitor the conversion of thymidine to thymine.
[b]The inhibition values were calculated from the percentage of thymidine remaining in the reaction mixture. The total conversion of dThd to Thy at the different time points (10 and 60 min) was taken as 100% to calculate the inhibition values. At 10 min and 60 min approximately 50% (55 μM) and 75% (73 μM) of thymidine was converted to thymine.

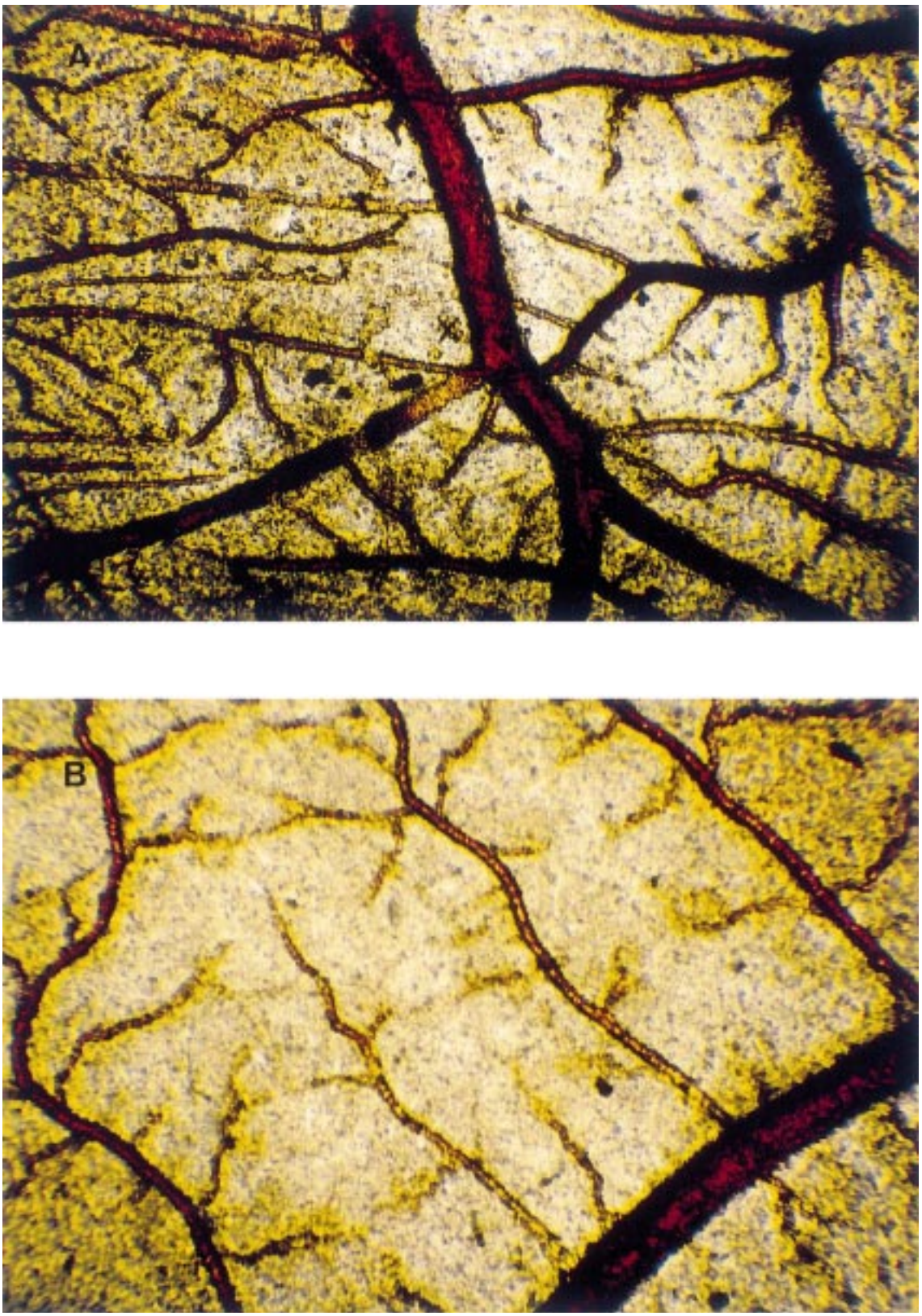

Fig. 4. Chorioallantoic membrane of fertilised chicken eggs in the absence (A) or presence (B) of 1 μmol 7DX.

observed in the CAM assay. These data provide additional proof of the importance of TPase as an angiogenic agent, and open interesting perspectives for the further exploitation of TPase inhibition (i.e. 7DX) as potential anticancer and anti-angiogenic agents.

A variety of modifications are now being introduced at the

pyrrole ring of 7DX in an attempt to optimise the lead compound as an inhibitor of TPase. These TPase inhibitors may prove useful in combination therapy with those compounds that have antiviral (i.e. BVDU, IdU, TFT) or antitumour (i.e. 5-FdU) potential but can be easily cleaved and thus inactivated by TPase. TPase (and UPase) inhibitors may also help in preventing the toxic side effects of 5-fluorouracil (5-FU) by suppressing the catabolism of 5-FdU (or 5-fluorouridine). It would now be interesting to evaluate whether TPase inhibitors such as 7DX favourably affect the antiviral and/or antitumour effects of these different dThd analogues.

The finding that 7DX has a marked anti-angiogenic activity in the chicken egg CAM assay also opens interesting perspectives for the further exploration of the TPase inhibitors as potential anti-cancer agents. Therefore, an extensive synthesis programme has now been generated to examine the potential of 7DX derivatives from both an antiviral and antitumour (i.e. anti-angiogenic) viewpoint, whether used alone (as single agents) or in combination with other chemotherapeutics.

Acknowledgements: We are grateful to Mrs Ria Van Berwaer for excellent technical assistance and Mrs Christiane Callebaut for fine editorial assistance. This research was supported by grants from the Biomedical Research Programme of the European Commission. R. Esnouf is a fellow of the Onderzoeksfonds of the Katholieke Universiteit Leuven. J. Neyts is a post-doctoral research assistant of the Fonds voor Wetenschappelijk Onderzoek–Vlaanderen.

References

[1] Desgranges, C., Razaka, G., Rabaud, M., Bricaud, H., Balzarini, J. and De Clercq, E. (1983) Biochem. Pharmacol. 32, 3583–3590.
[2] Furukawa, T., Yoshimura, A., Sumizawa, T., Haraguchi, M. and Akiyama, S.-I. (1992) Nature 356, 668.
[3] Sumazawa, T., Furukawa, T., Haraguchi, M., Yoshimura, A., Takeyasu, A., Ishizawa, M., Yamada, Y. and Akiyama, S. (1993) J. Biochem. 114, 9–14.
[4] Usuki, K., Saras, J., Waltenberger, J., Miyazono, K., Pierce, G., Thomason, A. and Heldin, C.H. (1992) Biochem. Biophys. Res. Commun. 184, 1311–1316.
[5] Haraguchi, M., Miyadera, K., Uemura, K., Sumizawa, T., Furukawa, T., Yamada, K., Akiyama, S. and Yamada, Y. (1994) Nature 368, 198.
[6] Moghaddam, A., Zhang, H.-T., Fan, T.-P.D., Hu, D.-E., Lees, V.C., Turley, H., Fox, S.B., Gatter, K.C., Harris, A.L. and Bicknell, R. (1995) Proc. Natl. Acad. Sci. USA 92, 998–1002.
[7] Miyadera, K., Sumizawa, T., Haraguchi, M., Yoshida, H., Konstanty, W., Yamada, Y. and Akiyama, S. (1995) Cancer Res. 55, 1687–1690.
[8] O'Brien, T.S., Fox, S.B., Dickinson, A.J., Turley, H., Westwood, M., Moghaddam, A., Gatter, K.C., Bicknell, R. and Harris, A.L. (1996) Cancer Res. 56, 4799–4804.
[9] Fox, S.B., Westwood, M., Moghaddam, A., Comley, M., Turley, H., Whitehouse, R.M., Bicknell, R., Gatter, K.C. and Harris, A.L. (1996) Br. J. Cancer 73, 275–280.
[10] Igarashi, M., Dhar, D.K., Kubota, H., Yamamoto, A., El-Assal, O. and Nagasue, N. (1998) Cancer 82, 1225–1232.
[11] Takebayashi, Y., Akiyama, S., Akiba, S., Yamada, K., Miyadera, K., Sumizawa, T., Yamada, Y., Murata, F. and Aikou, T. (1996) J. Natl. Cancer Inst. 88, 1110–1117.
[12] Toi, M., Hoshina, S., Taniguchi, T., Yamamoto, Y., Ishitsuka, H. and Tominaga, T. (1995) Int. J. Cancer 64, 79–82.
[13] Imazano, Y., Takebayashi, Y., Nishiyama, K., Akiba, S., Miyadera, K., Yamada, Y., Akiyama, S. and Ohi, Y. (1997) J. Clin. Oncol. 15, 2570–2578.
[14] Griffiths, L. and Stratford, I.J. (1997) Br. J. Cancer 76, 689–693.
[15] Langen, P., Etzold, G., Bärwolff, D. and Preussel, B. (1967) Biochem. Pharmacol. 16, 1833–1837.
[16] West, R.A., Ledig, K. and Hitchings, G.B., British Patent 812.366, 1959. [Chem. Abstr. 54, 592I (1960)].
[17] Schroeder, E.F., US Patent 2.731.465, Jan. 17, 1956. [Chem. Abstr. 51, 1257 (1957)].
[18] Maragoudakis, M.E., Panoutsakopoulou, M. and Sarmonika, M. (1988) Tissue Cell 20, 531–539.
[19] Harris-Hooker, S.A., Gajdusek, C.M., Wight, T.N. and Schwartz, S.M. (1983) J. Cell. Physiol. 114, 302–310.
[20] 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.