Anti-angiogenic activity of a novel multi-substrate analogue inhibitor of thymidine phosphorylase

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Abstract 7-Deazaxanthine (7-DX) was recently identified as the first purine derivative with pronounced inhibitory activity against *Escherichia coli* thymidine phosphorylase (TP) and angiogenesis. In order to 'freeze' the enzyme in an open, inactive conformation, a novel multi-substrate analogue inhibitor of TP, containing an alkyl phosphonate moiety covalently linked to 7-DX, was synthesized. The prototype compound TP65 (9-(8-phosphonooctyl)-7-deazaxanthine) (at 250 μM) completely inhibited TP-induced formation of microvascular sprouts from endothelial cell aggregates in a three-dimensional fibrin gel. In the chick chorioallantoic membrane assay, TP caused a dose-dependent stimulation of angiogenesis, which was completely inhibited by 250 nmol TP65. This dose proved to be non-toxic for the developing chick embryo. TP65 thus emerges as a potent and specific inhibitor of TP and TP-induced angiogenesis, which opens new perspectives for multi-substrate analogue inhibitors of TP as potential anti-cancer agents and as inhibitors of angiogenesis and of diseases with enhanced expression of TP.

Key words: 7-Deazaxanthine; Thymidine phosphorylase; Angiogenesis; Multi-substrate analogue inhibitor; 9-(8-Phosphonooctyl)-7-deazaxanthine; Endothelial cell

1. Introduction

Angiogenesis, the process of generating new capillary blood vessels from pre-existing vessels, is a fundamental process in reproduction [1,2] and wound healing [2]. In these conditions, neovascularization is tightly regulated. Unregulated angiogenesis may lead to several angiogenic diseases [3] and is thought to be indispensable for solid tumor growth and metastasis [4]. The construction of a vascular network requires different sequential steps including the release of proteases from 'activated' endothelial cells with subsequent degradation of the basement membrane, endothelial cell migration and proliferation, and differentiation into mature blood vessels. These processes are mediated and modulated by a wide range of angiogenic inducers, including growth factors, chemokines and enzymes [2].

Platelet-derived endothelial cell growth factor/thymidine phosphorylase (TP) is an enzyme with important angiogenic properties. TP has been shown to stimulate endothelial cell migration in vitro and angiogenesis in vivo [5–8]. The enzymatic activity of TP was found to be crucial for its angiogenic effect [9]. TP induces the reversible phosphorolysis of pyrimidine deoxynucleosides to 2-deoxyribose 1-phosphate and their respective pyrimidine bases [10]. Recent observations suggest that 2-deoxyribose 1-phosphate, which is a strongly reducing sugar, generates oxygen radical species, which may induce the secretion of oxidative stress-responsive angiogenic factors, like vascular endothelial growth factor, interleukin-8 and matrix metalloproteinase-1 [11].

TP is overexpressed in many solid tumors and TP levels correlate well with microvessel density in breast [12], ovarian [13], colorectal [14], endometrial [15] and oesophageal [16] cancers, pointing to a role for this enzyme in tumor vascularization. Moreover, TP has recently been shown to inhibit tumor cell apoptosis [14,17]. Therefore, there is an anti-cancer potential for potent and specific TP inhibitors. Among the first inhibitors of TP that have been described, 6-aminothymine and 6-amino-5-bromouracil are the most potent [18]. Based on the structure of *Escherichia coli* TP [19], which shows 40% sequence identity with human TP, we recently designed and synthesized the first purine derivative (7-deazaxanthine, 7-DX) with inhibitory activity against *E. coli* TP [20]. We used 7-DX as a lead compound to develop a novel type of multi-substrate analogue inhibitor of TP [21,22]. The prototype compound (9-(8-phosphonooctyl)-7-deazaxanthine, TP65) contains an alkyl phosphonate moiety, covalently linked to 7-DX. We proved that this novel TP inhibitor was able to interact at both the phosphate binding site and the nucleoside binding site, thus 'freezing' the enzyme in an open, inactive conformation [22].

In the present study, we describe the marked inhibitory activity of the novel multi-substrate analogue TP inhibitor (TP65) against angiogenesis induced by TP in both cell culture and in the chorioallantoic membrane (CAM) assay.

2. Materials and methods

2.1. Compounds

7-DX and the multi-substrate analogue inhibitor TP65 were synthesized as described [20,21]. For the chemical structures of these compounds, see Fig. 1. *E. coli* TP (1030 U/ml) was obtained from Sigma (St. Louis, MO, USA).

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2.2. Cells

Mouse brain endothelial (MBE) cells, provided by Dr. M. Presta (Brescia, Italy) were maintained in Dulbecco's modified minimum essential medium (DMEM; Gibco, Life Technologies, Rockville, MD, USA) supplemented with 2 mM glutamine (Gibco) and 10% fetal calf serum (Integro, Zaandam, The Netherlands).

2.3. Purification of glutathione-S-transferase (GST)-TP

The *E. coli* TP gene was expressed in *E. coli* as a GST fusion protein as follows. The TP gene was isolated from genomic DNA of *E. coli* K12 and subcloned in the pGEM-T vector (Promega, Madison, WI, USA). The forward (5′-AAG AAT TCT TTC TCG CAC AAG AAA TTA TTC G) and reverse (5′-AAG TCG ACT TAT TCG CTG ATA CGG CG) primers (Gibco, Paisley, UK) introduce an *Eco*RI and *Sal*I site, respectively. The TP gene was subsequently cloned between the *Eco*RI and *Sal*I sites of the pGEX-5X-1 vector (Amersham Pharmacia Biotech, Uppsala, Sweden). The resulting plasmid vector (pGEX-5X-1-TP) was checked by automated fluorescence sequencing (ALFexpress, Amersham Pharmacia Biotech) and transfected into *E. coli* BL21(DE3)pLysS. Bacteria were grown overnight at 37°C in 2YT medium containing ampicillin (100 μg/ml) and chloramphenicol (40 μg/ml), and then diluted 1:10 in fresh medium. After further growth of the bacteria at 27°C (for 1 h), isopropyl-β-D-thiogalactopyranoside (Sigma) was added to a final concentration of 0.1 mM to induce the production of the GST-TP fusion protein. After 15 h of further growth at 27°C, cells were pelleted (6000×*g* for 10 min at 4°C) and resuspended in lysis buffer (50 mM Tris, pH 7.5, 1 mM dithiothreitol, 5 mM EDTA, 10% glycerol, 1% Triton X-100, 0.1 mM phenylmethylsulfonylfluoride (PMSF) and 0.15 mg/ml lysosyme). Bacterial suspensions were homogenized and lysed by means of a 'French Pressure cell press', and ultracentrifuged (20 000×*g* for 15 min at 4°C). GST-TP was purified from the supernatant using Glutathione Sepharose 4B (Amersham Pharmacia Biotech) as described by the supplier. Briefly, a 50% slurry of Glutathione Sepharose was added to the bacterial supernatant (1.5 ml/1.5 l of broth), incubated for 30 min at 4°C, and then washed three times with 10 bed volumes (7.5 ml) of lysis buffer without lysosyme and PMSF. Bound proteins were eluted in 50 mM Tris (pH 8.0) containing 0.1% Triton X-100 and 10 mM glutathione. Protein content of the purified fractions was assessed using Bradford reagent (Sigma).

2.4. Endothelial cell-sprouting assay

Fibrinogen (5 mg/ml) was dissolved in DMEM/HAM12 medium. Then, MBE cell aggregates, prepared on agarose-coated plates (to prevent spreading of the cells), as described previously [23], were resuspended in the fibrinogen solution, and clotting was initiated by the addition of thrombin (500 mU/ml). The mixture was transferred into 24-well plates and allowed to gelify at 37°C. Cell aggregates were maintained for 3 days in DMEM/HAM12 medium containing the test compounds, and the formation of endothelial cell sprouts was evaluated daily.

2.5. CAM assay in fertilized chicken eggs

The in vivo CAM angiogenesis model was performed as described by Maragoudakis et al. [24]. Fertilized eggs were incubated for 3 days at 37°C when 3 ml of albumin was removed (to detach the shell from the developing CAM) and a window was opened on the eggshell exposing the CAM. The window was covered with cellophane tape and the eggs were returned to the incubator until day 9 when the test compounds were applied. The compounds were placed on sterile plastic discs (Ø 8 mm) which were allowed to dry under sterile conditions. A solution of cortisone acetate (100 μg/disc, Sigma) was added to all discs in order to prevent an inflammatory response. A loaded and dried control disc was placed on the CAM 1 cm away from the disc containing the test compound(s). Next, the windows were covered and the eggs incubated until day 11 when angiogenesis was assessed. At day 11, the eggs were flooded with 10% buffered formalin (Janssen Chimica, Geel, Belgium), the plastic discs were removed and the eggs were kept at room temperature for 2 h. A large area around the discs was cut off and placed on a glass slide, and the vascular density index was measured by the method of Harris-Hooker et al. [25]. Briefly, a grid containing three concentric circles of 4, 5 and 6 mm diameter was positioned on the surface of the CAM previously covered by the disc. Next all vessels intersecting the circles were counted. A two-tailed paired Student's *t*-test was used to assess the significance of the obtained results.

2.6. Inhibitory effect of TPase inhibitors on blood vessel formation induced by exogenous TPase

Angiogenesis in the CAM assay was stimulated by the addition of a commercial preparation of pure *E. coli* TP. Different amounts of the enzyme (20, 10, 5 or 1 U), spotted on discs, were applied onto the CAM. After 2 days, the number of blood vessels under each disc were counted and compared with discs that contained only phosphate-buffered saline (PBS) (or dimethyl sulfoxide, DMSO). To evaluate the effect of the inhibitors on TPase-induced blood vessel formation, TP65 and 7-DX (250 nmol) were spotted together with the enzyme (10 U) on the discs. Determination of blood vessel formation was performed after 2 days (i.e. day 11).

2.7. Cytostatic and cytotoxic effect of TP-65 and 7-DX in endothelial cell cultures

Endothelial cells from sponge implants (SIEC) [26] were seeded at two different cell densities (10 000 or 20 000 cells) per well of a gelatin-coated 96-well microtiter plate and exposed to 250, 100, 50, 20 and 5 μM of the test compounds in DMEM, supplemented with 1 mM L-glutamine, 1% non-essential amino acids, 1 mM sodium pyruvate and 20% FCS. Freshly prepared endothelial cell growth supplement and heparin at 100 μg/ml was also added to the medium. After 4 days of incubation, cells were trypsinized and counted by a Coulter counter (Harpenden Hertz, UK) or stained with trypan blue to reveal potential cellular toxicity of the compounds.

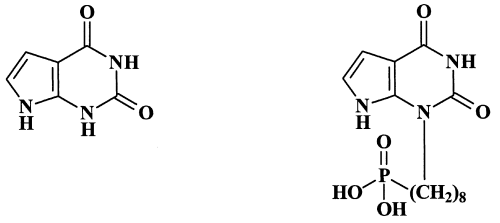
3. Results

3.1. Inhibition of tube formation in vitro by TP65 and 7-DX

TP65 and 7-DX were evaluated for their capacity to affect the sprouting of endothelial cell sprouts in vitro. In this assay, endothelial cell aggregates are embedded into a fibrin gel in the presence of an angiogenic stimulus, which induces the formation of endothelial cell sprouts. Accordingly, MBE cell aggregates were incubated in the presence of 100 U/ml of recombinant *E. coli* GST-TP. After 2 days of incubation, MBE cells started to invade the gel and to develop radially growing sprouts (Fig. 2B). TP65 (at 250 nM) and 7-DX (at 250 nM) completely inhibited the formation of endothelial sprouts induced by GST-TP (Fig. 2C for TP65 and data not shown for 7-DX).

3.2. Inhibitory effect of TP65 on neovascularization in the CAM assay

Based on our previous cell culture observations, we then evaluated the effect of TP65 on angiogenesis in the CAM assay. The in vitro tube formation experiment was performed with *E. coli* TP, purified as a fusion protein with GST. In order to rule out the possibility that GST might influence the activity of TP or TP65, we used a commercial preparation of pure *E. coli* TP to stimulate angiogenesis in the CAM assay.



7-Deazaxanthine (7-DX) **1-(8-phosphonooctyl)-7-deazaxanthine (TP65)**

Fig. 1. Structural formulae of 7-DX and the multi-substrate analogue inhibitor TP65.

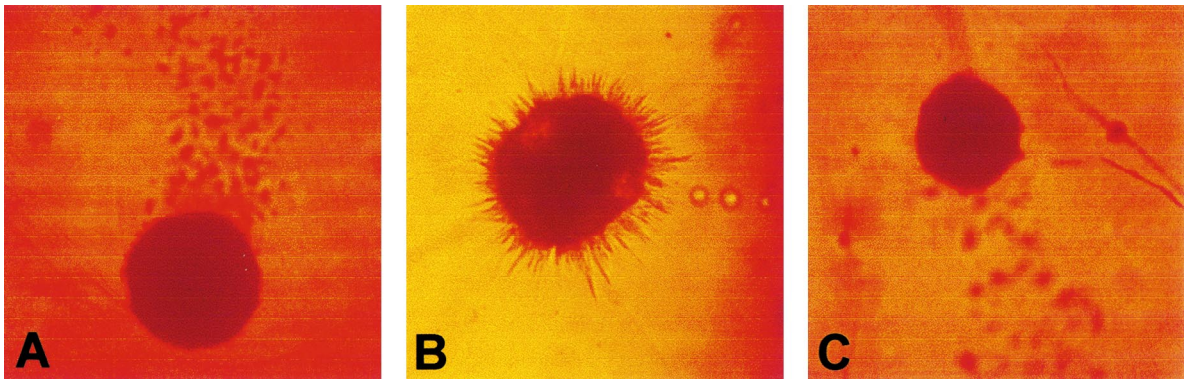


Fig. 2. Effect of TP65 on tube formation in vitro. Aggregates containing 25 000 MBE cells were suspended in a fibrinogen gel and incubated in DMEM/HAM12 medium. Control cultures showed migration of endothelial cells after 3 days (A). Addition of TP resulted in the formation of microvascular tubes (B). The angiogenic activity of TP was completely inhibited by the addition of 250 nmol TP65 (C). Original magnification: 40×.

In a first set of experiments, the effect of different TP amounts on blood vessel formation was investigated. After 2 days of exposure, TP caused a dose-dependent formation of new blood vessels that developed radially towards the disc containing the angiogenic stimulus (Fig. 3A). 10 U of TP induced angiogenesis in all CAMs tested, with an average stimulation of 40% ± 8 ($P < 0.05$, Fig. 3A–C). TP was clearly stimulatory at 5 U/disc (16% ± 11, $P < 0.05$), although only 60% of the CAMs showed an increase in the number of blood vessels (Fig. 3B). Application of 20 U of TP onto the CAM resulted in inflammation and the appearance of blood clots, which made morphological evaluation of the vessels virtually impossible. Therefore, 10 U of TP was considered to be the optimal dose to evaluate the activity of test compounds (i.e. TP65 and the reference compound 7-DX) in the CAM assay.

In a second set of experiments, TP65 and 7-DX were applied onto the CAM of fertilized chicken eggs at 50 and 250 nmol. At least 10 eggs were scored for each inhibitor concentration in two independent experiments. TP65 markedly prevented blood vessel formation, induced by TP, on the CAM during a 2-day exposure time period. At 250 nmol of TP65, a complete inhibition of TP-induced blood vessel formation was observed, i.e. 40% stimulation by TP in the absence of TP65 versus 5% inhibition by TP in the presence of TP65 ($P < 0.001$, Figs. 4A and 5B), and only 8% of the CAMs displayed an increase in the number of blood vessels (versus 100% in the CAMs exposed to TP alone, Fig. 4B). At 50 nmol of TP65, stimulation of angiogenesis by TP was not inhibited. 7-DX, dissolved in DMSO, was included as a reference compound. Although DMSO alone diminished the angiogenic effect of TP to 24% ± 9 (Fig. 4C), addition of 250 nmol of 7- DX further reduced neovascularization to 6% ± 11 ($P < 0.05$, Fig. 4C), with 65% of the CAMs showing stimulation (Fig. 4D). TP65 thus proved to be a markedly better inhibitor of TP-induced angiogenesis in the CAM assay than 7-DX.

3.3. *Cytostatic activity of TP65 and 7-DX against endothelial cells*

TP65 and 7-DX have been evaluated for their anti-proliferative and cytotoxic activity against endothelial (SIEC) cell cultures. At 250, 100 and 50 μM, the compounds did not

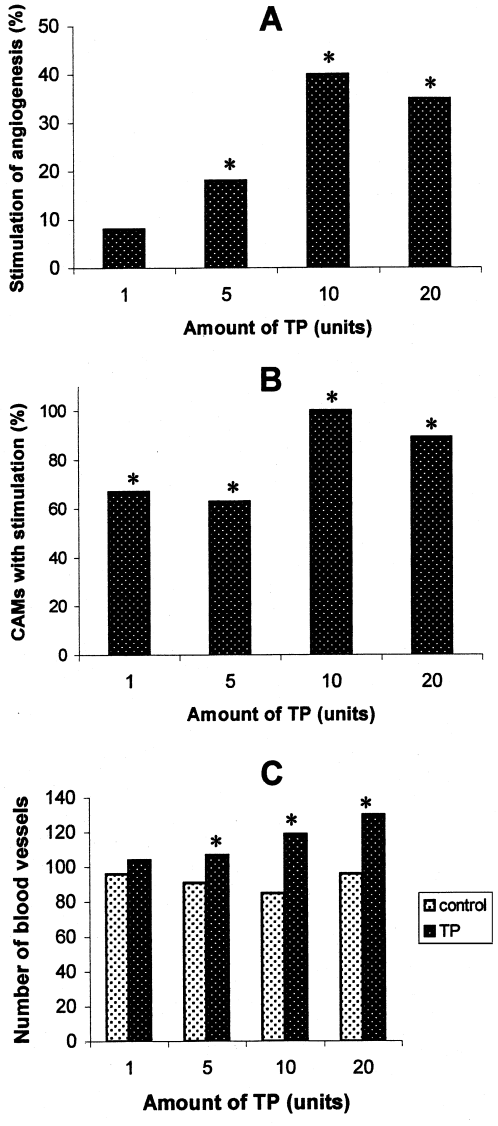


Fig. 3. Stimulation of angiogenesis by TP in the CAM assay. At day 9 of incubation, discs containing either TP or PBS were applied onto the CAM. At day 11, the blood vessels under each disc were counted and the percentage of stimulation (A), the percentage of the CAMs that show stimulation (B), and the number of blood vessels (C), were determined (*$P < 0.05$).

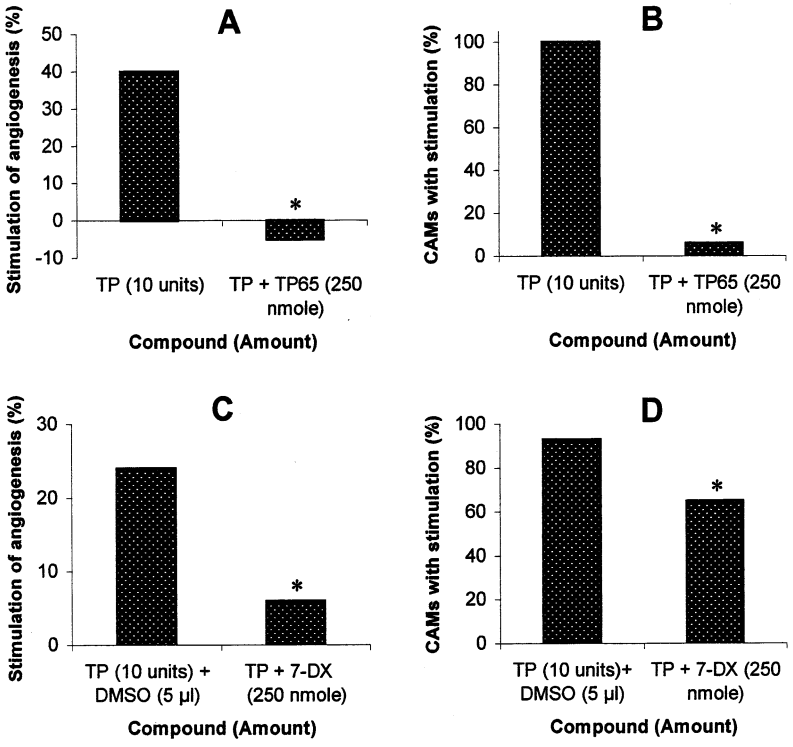


Fig. 4. Inhibition of TP-induced angiogenesis in the CAM assay by TP65 and 7-DX. At day 9 of incubation, discs containing either TP (A–D), TP plus TP65 (A, B) or TP plus 7-DX (C, D) were applied onto the CAM. At day 11, the percentage of the CAMs that show stimulation (B, D) and the percentage of stimulation (A, C), were determined ($*P < 0.05$).

inhibit endothelial cell proliferation. The cell numbers obtained after 4 days of drug exposure at the indicated compound concentrations were not significantly different from drug-free control cultures. Also, trypan blue dye exclusion revealed $>95\%$ viability at 250 and 100 μM of both test compounds upon a 3-day exposure time period.

4. Discussion

7-DX was identified as the first purine derivative with inhibitory activity against TP [20]. The compound may mimic thymine in the TP active site since it contains the CO–NH–CO functional entity of the pyrimidine ring, while the $C_7=C_8$ moiety of the pyrrole ring of 7-DX corresponds to the 5-methyl part of the thymine molecule. 7-DX was used as a lead compound to design more potent purine-based multi-substrate TP inhibitors, such as TP65 [21,22]. TP65 is a multi-substrate inhibitor, able to concomitantly interact at the phosphate binding site and the nucleoside binding site, with the purpose of immobilizing the enzyme in an open, inactive conformation [21,22].

TP65 completely abrogated the formation of vascular sprouts from endothelial cell aggregates in vitro. In the CAM assay, both the percentage of CAMs in which TP

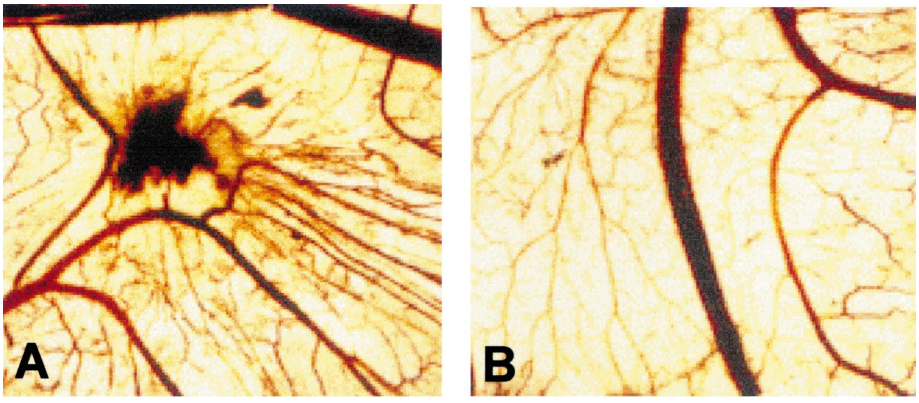


Fig. 5. Effect of TP65 on TP-induced angiogenesis in the CAM assay. At day 9 of incubation, discs containing either TP or TP plus TP65 were applied onto the CAM. Two days later, new blood vessels developed radially towards the disc containing TP (A), whereas no increase in the number of blood vessels or a change in their orientation could be observed when the disc contained TP plus TP65 (B).

causes stimulation of angiogenesis and the increase in the number of blood vessels were determined. A maximum stimulation of 40% could be achieved by 10 U of *E. coli* TP. However, the method of Harris-Hooker [25] does not distinguish between newly formed microvessels (after application of the discs on the CAM) and those that were already present at day 9 and, therefore, may underestimate the stimulatory activity of TP.

In the CAM assay, 7-DX partially inhibited TP-induced angiogenesis, under the experimental conditions where the novel multi-substrate analogue inhibitor TP65 completely abrogated the formation of TP-induced neovascularization of the chick CAM. Thus, angiogenesis induction by TP was completely inhibited by the addition of 250 nmol of TP65. Furthermore, TP65 did not cause embryo death or inflammation of the CAM at the doses used in this study, nor did it inhibit the proliferation of endothelial cells in vitro, which clearly indicates that the inhibition of TP-induced angiogenesis is a specific, non-toxic effect.

Angiogenesis is induced by a variety of molecules, many of them are peptide growth factors, which interact with two types of receptors: low affinity receptors or heparin-containing glycosaminoglycans and high affinity tyrosine kinase receptors [2]. Heparin binding occurs via basic amino acid residues in the growth factor molecule. All known inhibitors of these growth factors either disrupt the binding of the growth factor to heparin or to the tyrosine kinase receptor. In contrast, TP is not a growth factor, i.e. it does not stimulate endothelial cell proliferation, and does not interact with any receptor. In fact, the angiogenic action of TP depends on its enzymatic activity and the subsequent release of 2-deoxy-D-ribose [3,7], which is the molecule responsible for the angiogenic effect of TP [27]. Thus, the mechanism of action and the structure of TP65 is totally different from those of heparin-binding growth factors. Since TP65 has been specifically designed as a multi-substrate inhibitor, based on the structure of *E. coli* TP, and since this compound was proven to concomitantly interact with two different sites of the TP enzyme [22], it is very unlikely that this TP inhibitor also affects the activity of angiogenic growth factors.

Since both GST-TP and commercially available TP are produced in *E. coli*, they may contain endotoxins. However, we are convinced that the angiogenic activity of GST-TP and TP is not due to endotoxin (known to be an angiogenic agent) contamination because of a lack of angiogenic activity has been reported by Moghaddam et al. [8] for a mutated TP enzyme also produced in *E. coli*. Moreover, the angiogenic activity of GST-TP and TP in the present study can be completely inhibited by the TP inhibitor TP65, which would be impossible if blood vessel formation was caused by contaminating endotoxin.

Taken together, TP65 emerges as a highly specific and non-toxic TP inhibitor. Although the in vivo activity of TP65 still has to be established, our findings open new perspectives for the use of this novel type of multi-substrate analogue inhibitor in the treatment of cancer and various other angiogenic diseases that overexpress TP, such as rheumatoid arthritis [27], psoriasis [28], atherosclerosis [29] and gastric ulcers [30]. In addition, TP65 may also potentiate the activity of several antiviral agents (i.e. (*E*)-5-(2-bromovinyl)-2′-deoxyuridine, 5-iodo-2′-deoxyuridine) and anti-tumor agents (i.e. 5-fluoro-2′-deoxyuridine) that are susceptible to inactivation (hydrolysis) by TP [31,32].

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