5′-O-Tritylated Nucleoside Derivatives: Inhibition of Thymidine Phosphorylase and Angiogenesis

Sandra Liekens, Annelies Bronckaers, Ana-Isabel Hernández, Eva-María Priego, Elena Casanova, Maria-José Camarasa, Maria-Jesus Pérez-Pérez, and Jan Balzarini

Rega Institute for Medical Research, Katholieke Universiteit Leuven, Leuven, Belgium (S.L., A.B., J.B.); and Instituto de Química Médica, Madrid, Spain (A.-I.H., E.-M.P., E.C., M.-J.C., M.-J.P.-P.)

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ABSTRACT

Thymidine phosphorylase (TPase) is one of the key enzymes involved in the pyrimidine nucleoside salvage pathway. However, TPase also stimulates angiogenesis, and its expression correlates well with microvessel density and metastasis in a variety of human tumors. We have shown recently that 5′-O-trityl-inosine (KIN59) allosterically inhibits TPase enzymatic activity. KIN59 also inhibits TPase-induced angiogenesis in the chick chorioallantoic membrane (CAM) assay. The trityl group was found to be instrumental to preserve both the anti-TPase and antiangiogenic effect. We have now synthesized a variety of novel 5′-O-trityl nucleoside derivatives. Enzyme activity studies showed that the anti-TPase activity is significantly improved by replacement of the hypoxanthine base by thymine [3.5-fold; i.e., 5′-O-tritylthymidine (KIN6)] and the introduction of chloride on the trityl group [7-fold; i.e., 5′-O-(4-chlorotrityl)-inosine (TP136)], whereas removal of 2′-hydroxyl in the ribose did not significantly alter the anti-TPase activity. Enzyme kinetic studies also demonstrated that 1-(5′-O-trityl-β-D-ribofuranosyl)-thymine (TP124), like KIN59, inhibits TPase in a noncompetitive fashion both with respect to phosphate and thymidine. Most KIN59 analogs markedly inhibited TPase-induced angiogenesis in the CAM assay. In vitro studies showed that the antiangiogenic effect of these compounds is not attributed to endothelial cell toxicity. For several compounds, there was no stringent correlation between their anti-TPase and antiangiogenic activity, indicating that these compounds may also act on other angiogenesis mediators. The antiangiogenic 5′-O-trityl nucleoside analogs also caused degradation of pre-existing, immature vessels at the site of drug exposure. Thus, 5′-O-trityl nucleoside derivatives combine antiangiogenic and vascular-targeting activities, which opens perspectives for their potential use as anticancer agents.

Angiogenesis is the formation of new capillaries from pre-existing blood vessels. It is a complex multistep process that requires an extensive interplay between a multitude of cellular and soluble factors with either inhibitory or stimulatory functions (Liekens et al., 2001a). The formation of an intra-tumoral network of blood vessels is required to provide the growing tumor with oxygen and nutrients. In addition, tumor neovascularization prevents tumors from undergoing necrosis and apoptosis and facilitates the escape of tumor cells into the circulation and subsequent metastasis to distant organs (Liekens et al., 2001a).

In 1987, platelet-derived endothelial cell growth factor (PD-ECGF) was isolated from human platelets and later from placenta tissue (Miyazono et al., 1987; Usuki et al., 1990). PD-ECGF was found to induce [3H]thymidine incorporation and endothelial cell migration in vitro and angiogenesis in vivo (Ishikawa et al., 1989). Later it was shown that PD-ECGF is identical with thymidine phosphorylase

ABBREVIATIONS: PD-ECGF, platelet-derived endothelial cell growth factor; CAM, chick chorioallantoic membrane; dThd, thymidine; MAEC, mouse aortic endothelial cell; HPLC, high-pressure liquid chromatography; TPase, thymidine phosphorylase; VEGF, vascular endothelial growth factor; TP146, 5′-O-(4,4′-dimethoxytrityl)inosine; TP134, 5′-O-trityluridine; KIN6, 5′-O-tritylthymidine; TP136, 5′-O-(4-chlorotrityl)-inosine; D7154, 5′-O-(4,4′-dimethoxytrityl)-2′-deoxyguanosine; TP140, 5′-O-trityl-1-methylinosine; TP65, 9-(8-phosphonooctyl)-7-deazaxanthine; TA-01, 5′-O-trityladenosine; KIN59, 5′-O-trityl-inosine; TP151, 5-amino-1-(5′-O-trityl-β-D-ribofuranosyl)imidazole-4-[N-(2-methoxyethoxy)methyl] carboxamide; buffer B, NaH2PO4 and heptane sulfonic acid; SU-6668, ((Z)-3-(2,4-dimethyl-5-(2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-1H-pyrrol-3-yl)propionic acid; TP137, 5′-O-trityl-2′-deoxy-5-methylcytidine; TP141, 5′-O-trityl-6-methyladenosine; TP142, 1-methyl-5′-O-trityl-β-D-ribofuranoside; TP124, 1-(5′-O-trityl-β-D-ribofuranosyl)thymine; TP147, 1-(5′-O-(4,4′-dimethoxytrityl)-β-D-ribofuranosyl)thymine.

(TPase). TPase catalyzes the reversible phosphorolysis of thymidine (dThd) and related analogs to 2-deoxy-D-ribose-1-phosphate and their respective bases. 2-Deoxy-D-ribose-1-phosphate is quickly dephosphorylated to 2-deoxy-D-ribose, which freely diffuses out of the cell. It has been proposed that 2-deoxy-D-ribose is essential for the angiogenic activity of TPase (Haraguchi et al., 1994; Miyadera et al., 1995; Moghaddam et al., 1995). More recently, TPase was also found to protect cells from apoptosis induced by hypoxia, Fas, or cisplatin (Ikeda et al., 2002, 2003; Mori et al., 2002). The mechanism of action is under debate, and it is not clear whether the enzymatic activity of TPase is required for inhibition of apoptosis.

Western blot (and histological) analyses of a variety of human tumors showed up to 10-fold higher expression of TPase in the tumors compared with the corresponding non-neoplastic regions of the same organs. In the majority of these studies, increased TPase levels correlated well with angiogenesis, invasion, metastasis, and shorter patient survival (Toi et al., 1995; Matsuura et al., 1999; Sivridis et al., 2002; Toi et al., 2005). Besides tumor cells, also lymphocytes, fibroblasts, and tumor-infiltrating macrophages were found to express elevated levels of TPase (Sivridis et al., 2002; Akiyama et al., 2004; Toi et al., 2005). In fact, macrophages are known to produce multiple angiogenesis mediators, including cytokines that may up-regulate TPase transcription and enzyme activity, such as tumor necrosis factor-α, interleukin-1, interferon-γ, and interferon-α (Goto et al., 2001; Zhu and Schwartz, 2003; De Bruin et al., 2004; Yao et al., 2005). In addition, hypoxia, low pH, and chemotherapeutic agents, including cyclophosphamide, oxaliplatin and taxanes, and X-ray radiation have been shown to increase TPase/PD-ECGF protein levels in various human tumors (Griffiths et al., 1997; Sawada et al., 1999; Kikuno et al., 2004, Toi et al., 2004, 2005).

TPase thus protects cells from apoptosis induced by various cell-damaging agents, which has lead to the hypothesis that a combination of TPase-inducible chemotherapy with TPase-targeted treatment, such as fluorouracil derivatives, might enhance the effectiveness of anticancer therapy (Kikuno et al., 2004, Toi et al., 2004). A recent phase III trial on metastatic breast cancer has indeed shown that the addition of capecitabine to standard TPase-inducible chemotherapy like paclitaxel, cisplatin, cyclophosphamide, or irradiation results in increased response rate, time to progression, and survival of patients compared with standard treatment alone (Toi et al., 2005; Walko and Lindley, 2005). Also TPase inhibitors with potent antiangiogenic activity may improve future TPase-targeted therapy. At this moment, there are no TPase inhibitors clinically available, although several drugs have been tested preclinically and clinically (Pérez-Pérez et al., 2005). Since 1998, our research groups have been involved in the search for novel TPase inhibitors. We have described 7-deazaxanthine as the first purine derivative able to inhibit TPase (Balzarini et al., 1998), and TP65 as the first multisubstrate inhibitor of TPase (Balzarini et al., 2000; Esteban-Gamboa et al., 2000; Pérez-Pérez et al., 2005). These compounds also inhibited angiogenesis in the chick chorioallantoic membrane (CAM) assay (Balzarini et al., 1998; Liekens et al., 2002). Recently we reported the inhibitory activity of the inosine analog 5′-O-trityl-inosine (KIN59) against human and bacterial TPase (Liekens et al., 2004).

The compound has unique features as a TPase inhibitor: it contains a purine base (hypoxanthine), a ribose sugar, and a trityl moiety at the 5′-position of the sugar. The trityl group of KIN59 is essential for both its inhibitory activity against TPase and in the CAM assay (Liekens et al., 2004). Moreover, in contrast to all TPase inhibitors described previously, KIN59 does not compete with nucleosides or phosphate for binding to the enzyme and is far more potent than other TPase inhibitors in the CAM assay (Liekens et al., 2004). In fact, KIN59 completely inhibited the formation of new blood vessels induced by TPase on the CAM without being toxic to the developing chick embryo. These observations indicate that the angiogenic activity of TPase might also be regulated by an as-yet-unidentified allosteric site in the enzyme.

To gain more insight into the structure-activity relationship of 5′-O-tritylated nucleosides as inhibitors of TPase and angiogenesis, and in an attempt to improve their anti-TPase activity, we synthesized a variety of KIN59 derivatives with modifications on the base (i.e., hypoxanthine, thymine, uracil, 5-methylcytosine), sugar (i.e., ribose, 2′-deoxyribose), and trityl (i.e., chlorotrityl, dimethoxytrityl) moieties. Here, we describe the activity of these compounds against TPase enzymatic activity, endothelial cell migration and proliferation in vitro, and TPase-induced angiogenesis in vivo. We also provide evidence that 5′-O-tritylated nucleosides combine antiangiogenic and vascular-targeting activities.

Materials and Methods

Compound Synthesis. KIN59 was synthesized as described previously (Liekens et al., 2004). TP146, TP134, TP124, TP147, and KIN6 have been prepared following described procedures (Munson, 1968; Tipson, 1968; Lewis et al., 1993; Beaton et al., 1998; Ewing et al., 2003). The synthesis of TP136, TP140, TP137, TP141, and TP151 will be reported elsewhere (E. Casanova, A.-I. Hernández, E.-M. Priego, S. Liekens, M.-J. Camarosa, J. Balzarini, and M.-J. Pérez-Pérez, unpublished data). 5′-O-Trityladenosine (TA-01) was synthesized by Dr. D. D. Petkov (Sofia, Bulgaria). The chemical structures of the compounds are shown in Fig. 1.

Cell Cultures. Mouse aortic endothelial cells (MAECs) were kindly provided by Professor M. Presta (Brescia, Italy). The cells were grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Harlan Sera-Lab Ltd., Loughborough, UK).

Purification of Recombinant TPase. The pMOAL-10T vector (Moghaddam and Bicknell, 1992) containing the human TPase gene (fused to glutathione S-transferase) was kindly provided by Professor R. Bicknell (Oxford, UK). Protein purification was performed as described previously (Liekens et al., 2002).

TPase Enzyme Assays. The phosphorolysis of thymidine (dThd) by human TPase was measured by high-pressure 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 dThd in the presence of 0.025 U of 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 heated at 95°C for 5 min. Next, the samples were rapidly cooled on ice, dThd was separated from thymine and quantified in the samples on a reversed-phase RP-8 column (Merck, Darmstadt, Germany) by HPLC analysis. The separation of thymine and dThd was performed by a linear gradient from 98% buffer B (50 mM NaH$_2$PO$_4$ and 5 mM heptane sulfonic acid, pH 3.2) and 2% acetonitrile to 50% buffer B and 50% acetoni-

trile. Retention times of thymine and dThd were 4.2 and 8.5 min, respectively. UV-based detection of thymine and dThd was performed at 267 nm.

To evaluate the inhibitory effect of the compounds, a variety of inhibitor concentrations were added to the reaction mixtures (500 μl) containing 100 μM 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 analyzed by HPLC.

In the kinetic assays, in which the inhibitory effect of TP124 was evaluated at varying concentrations of inorganic phosphate (P_i), TP124 was tested at concentrations ranging from 100 to 400 μM in the presence of 2, 3, 5, 10, and 20 mM P_i. The dThd concentration was kept fixed at 1000 μM. The reaction mixtures (100 μl) were then incubated for 20 min with 0.005 U of TPase, after which the tubes were heated to 95°C before cooling and HPLC analysis. In the kinetic assays in which the inhibition of TPase enzymatic activity was evaluated at varying concentrations of dThd, TP124 was tested at concentrations ranging from 100 to 400 μM in the presence of 125,

250, 500, 750, and 1000 μM dThd. The concentration of inorganic phosphate was kept constant at 25 mM. The analysis of the dThd-to-thymine conversion was performed as described above.

Evaluation of the Anti-TPase Activity of 5′-*O*-Tritylated Nucleoside Analogs in Intact Human Platelets. Freshly isolated blood platelets were obtained from the blood transfusion center of the University Hospital (Leuven, Belgium). The platelets were suspended in 25% plasma that had been diluted with platelet additive solution (2.94 g of sodium citrate, 4.08 g of sodium acetate, and 6.75 g of NaCl in 1 liter of H_2O, pH 7.2). The incubation mixture (1 liter) contained 500×10^6 blood platelets (in 500 μl), 400 μl of TPase assay buffer (see above), 100 μM dThd, and different concentrations of KIN59 or TP142. Incubations were performed at 37°C. At different time points (i.e., 0, 60, 120, and 180 min), 200-μl aliquots were withdrawn, transferred to Eppendorf tubes, and heated at 95°C for 3 min. Next, the samples were rapidly cooled on ice; dThd was separated from thymine and quantified by HPLC analysis as described above.
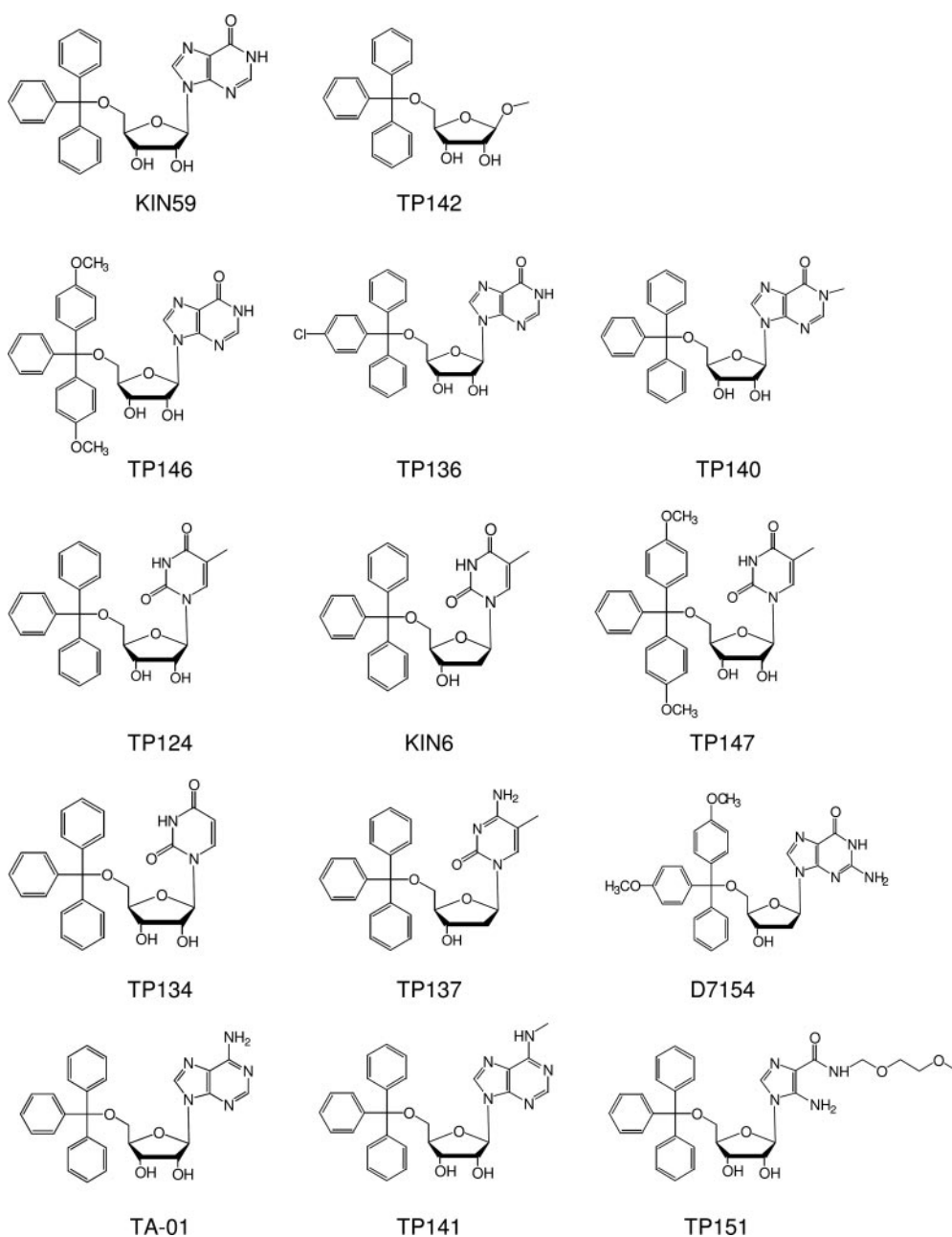

Fig. 1. Chemical structure of KIN59 and its derivatives.

Stability of the Compounds in CEM Cell Extracts. Exponentially growing CEM cell suspensions (50×10^6) were washed, resuspended in 500 μl of phosphate-buffered saline, and sonicated. Next, the cell extracts were cleared by centrifugation at 15,000g for 15 min at 4°C. One hundred microliters of CEM cell extract was incubated at 37°C in a total volume of 250 μl containing 50 μM concentrations of the different test compounds (KIN6, KIN59, and TP151). After 0 and 24 h, 100-μl aliquots were withdrawn from the reaction mixture, 200 μl of ice-cold MeOH was added, and the samples were incubated on ice and cleared by centrifugation. Stability of the compounds in the cell extracts was determined by HPLC analysis.

Cell Proliferation and Toxicity Assays. MAEC were seeded in 48-well plates at 10,000 cells/cm². After 16 h, cells were incubated in fresh medium in the presence of the test compounds, as indicated under *Results*. On day 5, cells were trypsinized and counted by a Coulter counter (Analis, Suarlée, Belgium).

Cell Wounding Assays. Wounds were created in confluent MAE cell monolayers with a 1.0-mm wide micropipette tip. Then, cells were incubated in fresh medium containing 10% fetal calf serum in the presence of the test compounds. After 16 h, the wounds were photographed, and endothelial cells invading the wound were quantified by computerized analysis of the digitized images.

CAM Assay in Fertilized Chicken Eggs. The in vivo CAM angiogenesis model was performed as described with slight modifications (Liekens et al., 2001b). Fertilized eggs were incubated for 3 days at 37°C when 3 ml of albumen 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 compounds were applied. The compounds were placed on sterile plastic discs (diameter, 8 mm), which were allowed to dry under sterile conditions. A solution of cortisone acetate (100 μg/disc; Sigma, St. Louis, MO) was added to all discs to prevent an inflammatory response. A loaded and dried control disc was placed on the CAM approximately 1 cm away from the disc containing the test compound(s). Next, the windows were covered, and the eggs were further incubated until day 11 when angiogenesis was assessed. Therefore, the membranes were fixed with 7% buffered formalin (Janssen Chimica, Geel, Belgium) and the plastic discs were removed. After fixation, a large area around the discs was cut off and placed on a glass slide. To determine the number of blood vessels, a grid containing two concentric circles with diameters of 3 and 5 mm was positioned on the surface of the CAM that was covered previously 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.

Results

Activity of 5′-*O*-Tritylated Nucleoside Derivatives against Human Recombinant TPase. We have shown previously that KIN59 inhibits human TPase in a noncompetitive fashion with respect to dThd and phosphate (Liekens et al., 2004). The presence of the trityl group was found to be important for the inhibitory activity. To gain more insight into the structural requirements that confer inhibitory activity to KIN59 and in an attempt to improve the anti-TPase activity, we synthesized a variety of 5′-*O*-tritylated nucleoside analogs (Fig. 1).

The anti-TPase activity of the prototype compound KIN59 increased 7-fold by the introduction of a chloro substituent on the trityl group (i.e., the IC_{50} value decreased from 30 μM for KIN59 to 4.5 μM for TP136) (Table 1). A substituent like methoxy on the trityl group, as in TP146, TP147, and D7154 (Fig. 1) resulted in instability of the compound upon prolonged incubation, and therefore, no reliable inhibition data could be obtained. Methylation of the hypoxanthine purine base (TP140) did not improve the inhibitory activity of the compound against TPase. Indeed, TP140 inhibited the TPase-catalyzed conversion of dThd to thymine at an IC_{50} value of 49 μM, compared with 30 μM for the parent compound KIN59 (Table 1).

The anti-TPase activity was also markedly improved by replacement of the purine base hypoxanthine by the pyrimidine base thymine (i.e., an IC_{50} value of 8.6 μM for KIN6 versus 30 μM for KIN59). Substitution of hypoxanthine by uracil (TP134) or 5-methylcytosine (TP137) slightly changed the anti-TPase activity of the compounds (i.e., IC_{50} values of 18 and 55 μM for TP134 and TP137, respectively). Comparison of KIN6 (bearing a 2′-deoxyribose) ($IC_{50} = 8.6$ μM) with TP124 (bearing a ribose) ($IC_{50} = 12$ μM) showed that the 2′-hydroxyl group is not very important for anti-TPase activity (Table 1).

Replacement of hypoxanthine (in KIN59) by the purine base adenine (TA-01) or 6-methyladenine (TP141) resulted in compounds that were highly instable, making determination of the anti-TPase activity virtually impossible.

TP151, which does not contain an intact purine or pyrimidine base but a structurally related heterocyclic base, or TP142, in which the base has been removed, did not inhibit the enzymatic activity of TPase ($IC_{50} > 70$ μM) (Table 1). Because of their limited solubility, TP151 and TP142 could not be evaluated in the TPase enzyme assays at concentrations greater than 70 μM. The enzyme activity studies demonstrate that flexibility in the nature of the purine or pyrimidine base is allowed, but the base cannot be entirely

TABLE 1

Effects of 5′-*O*-tritylated nucleoside analogs on TPase enzymatic activity and TPase-induced angiogenesis in the CAM assay

Values are presented as mean $\pm$ S.E.M.

Compound		TPase Enzyme Assay IC_{50}	CAM Assay[a] (250 nmol)
		μM	% of inhibition
KIN59	5′-*O*-Trityl-inosine	30 ± 8.7[a]	-89 ± 10
TP136	5′-*O*-(4-Chlorotrityl)-inosine	4.5 ± 1.6	-89 ± 6
TP140	5′-*O*-Trityl-1-methylinosine	49 ± 2.5	$+3 \pm 18$
TP124	1-(5′-*O*-Trityl-β-D-ribofuranosyl)thymine	12 ± 1.6	-28 ± 24
KIN6	5′-*O*-Trityl-thymidine	8.6 ± 0.8	-27 ± 16
TP134	5′-*O*-Trityl-uridine	18 ± 0.5	-76 ± 7
TP137	5′-*O*-Trityl-2′-deoxy-5-methylcytidine	55 ± 9.2	-92 ± 4
TP142	1-Methyl-5′-*O*-trityl-β-D-ribofuranoside	≥ 70	$+11 \pm 17$
TP151	5-Amino-1-(5′-*O*-trityl-β-D-ribofuranosyl)imidazole-4-[*N*-(2-methoxyethyl)methyl] carboxamide	>75	-67 ± 8

[a] Number of blood vessels of untreated controls, 25.7 ± 2.9.

removed, indicating that a (hetero)cyclic ring system must be present to keep inhibitory activity against TPase.

To reveal whether 5′-O-trityl derivatives are also inhibitory against TPase in intact cells, we next evaluated the anti-TPase activity of KIN59 and TP142 in intact human platelets, which contain high amounts of TPase (Desgranges et al., 1983). Therefore, 100 μM concentration of thymidine was added to freshly isolated human blood platelets in the presence of different concentrations of KIN59 or TP142 (Fig. 2). In the absence of the test compounds, 40, 62, and 75% of thymidine were converted to thymine after, respectively, 1, 2, or 3 h of incubation. KIN59 inhibited the conversion of dThd to thymine in a dose- and time-dependent manner, with a maximum inhibition of 53% after 1 h at 7.5 μM. In contrast, TP142, which did not inhibit TPase enzymatic activity in a cell-free system (up to 70 μM), had no inhibitory effect on TPase activity in blood platelets. These data indicate that the anti-TPase activity of the compounds in a cell-free system is also relevant for their activity in intact platelets, and that 5′-O-tritylated nucleoside analogs are taken up by intact platelets, are stable in intact cells under the experimental conditions, and exert their anti-TPase activity intracellularly.

We showed before that KIN59 inhibits TPase in a noncompetitive fashion, indicating that this compound does not bind to the substrate-binding sites of TPase. Among the different nucleoside analogs evaluated here, we chose TP124 to perform extensive kinetic enzyme studies. As shown in Fig. 3, TP124 (in which the hypoxanthine base in KIN59 was replaced with thymine) did not competitively interact with the dThd or phosphate-binding sites of TPase when dThd or phosphate was used as the variable substrate. Instead, TP124 seems to bind to the enzyme independently from the substrate in a mutually nonexclusive manner. These findings indicate that TP124 may interact with an allosteric site in the enzyme, a finding that also has been shown previously for KIN59.

Effect of 5′-O-Tritylated Nucleoside Derivatives on TPase-Induced Angiogenesis in the CAM Assay. To assess whether the anti-TPase activity of the KIN59 analogs correlates with their antiangiogenic effect, we evaluated these compounds in the CAM assay (Table 1 and Fig. 4). Local administration of 10 μl (10 units) of purified recombinant *Escherichia coli* TPase stimulated blood vessel formation in the CAM of fertilized chicken eggs by 24 $\pm$ 10% (Fig.

4A). Addition of 250 nmol of KIN59 resulted in a virtually complete inhibition of angiogenesis. Because angiogenesis is quantified by counting all blood vessels intersecting concentric circles with diameters of 3 and 5 mm, 100% inhibition reflects an avascular zone with a diameter of 5 mm. Not only TPase-induced angiogenesis but also normal blood vessel development was completely abrogated in all (100%) of the evaluated eggs (Figs. 4B and 5). The inosine analog TP136 also markedly inhibited TPase-induced neovascularization in all eggs at 250 nmol (Fig. 4, A and B). It is surprising that TP140 did not significantly affect TPase-induced blood vessel formation at 250 nmol (Fig. 4).

Among the nucleoside analogs containing uracil or thymine as the base part, TP134 caused a large avascular zone at 250 nmol, whereas exposure to TP124 and KIN6 resulted in a small avascular spot in almost 40% of the treated CAMs (Figs. 4B and 5). TP142, which did not possess anti-TPase activity, lacked significant activity in the CAM assay. However, the cytosine analog TP137 with modest anti-TPase activity proved to be a very potent inhibitor of TPase-induced angiogenesis at 250 nmol/disc, causing a large avascular zone in all evaluated CAMs. More surprisingly, TP151, which did not inhibit the enzymatic activity of TPase, was shown to be a potent inhibitor of TPase-induced angiogenesis (Figs. 4 and 5).

Cytostatic and Cytotoxic Effect on Vascular Endothelial Cells. Although we did not observe any visible toxicity to the chick embryos after exposure of the CAMs to the different compounds at the doses used, any compound that is toxic to endothelial cells is likely to possess an unspecified antiangiogenic effect. To evaluate whether the inhibition of CAM angiogenesis can, in part, be explained by a direct toxic
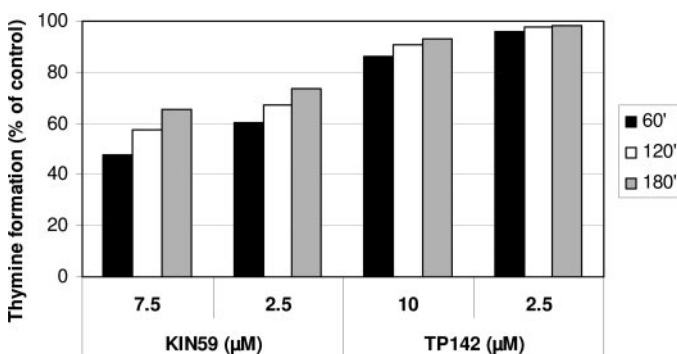

Fig. 2. Effects of 5′-O-tritylated nucleoside analogs on TPase activity in intact human platelets. Human blood platelets were incubated for 60, 120, or 180 min at 37°C in the presence of 100 μM dThd. The conversion of dThd to thymine in the absence or presence of different concentrations of KIN59 or TP142 was determined by HPLC.
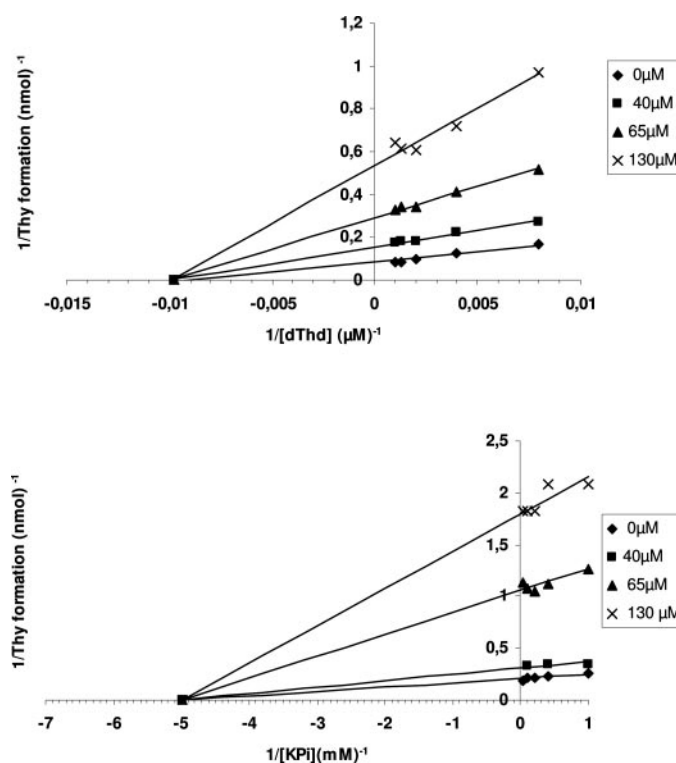

Fig. 3. Lineweaver-Burk plots of human TPase inhibition by TP124, in the presence of variable concentrations of dThd (top) and phosphate (bottom). KPi, concentration of potassium phosphate.

effect of the nucleoside analogs on endothelial cells, we performed endothelial cell proliferation and toxicity studies on confluent cell cultures. Therefore, we chose to investigate two compounds with a close correlation between anti-TPase and antiangiogenic activity (i.e., KIN59 and TP142) and two compounds with no apparent correlation (i.e., KIN6 and TP151). As shown in Table 2, KIN59 and TP151, which markedly inhibited angiogenesis in the CAM assay, poorly inhibited MAEC proliferation (IC_{50} values of 78 and 60 μM, respectively). In contrast, KIN6, which only caused a moderate inhibition of angiogenesis, inhibited endothelial cell proliferation at 9-fold lower concentrations ($IC_{50} = 8 \mu$M) and proved to be toxic to confluent endothelial cells at 100 μM (data not shown). Thus, inhibition of angiogenesis by KIN59 or TP151 could not be attributed to an effect on endothelial cell proliferation or cell toxicity, whereas the relative cell toxicity of KIN6 is clearly not sufficient to confer a potent antiangiogenic effect to the compound.

Endothelial Wound-Healing Assay. Next, we evaluated the effects of the tritylated nucleoside analogs on endothelial cell migration, which represents an important step in the formation of new capillaries. To this point we used an in vitro wound-healing assay using MAECs. Wounds were created in confluent cell monolayers with a 1.0-mm wide tip, after which control cells spontaneously migrate into the empty space. After 16 h, the extent of wound closure and the inhibition of cell migration by the compounds was determined by computerized imaging of the digital pictures (Fig. 6).

KIN59 and KIN6 dose-dependently inhibited MAEC migration, whereas TP151 only caused a delay in the recovery of the monolayer at the highest nontoxic dose used. In contrast, TP142 had no effect on the migration of the endothelial cells (Fig. 6 and Table 2).

Discussion

Based on previous results with KIN59, we hypothesized that 5′-O-tritylated nucleoside analogs may bind to an as-yet-unidentified allosteric site in TPase, thereby inhibiting TPase enzymatic activity and TPase-induced angiogenesis (Liekens et al., 2004). We showed that the trityl group is

important for both the anti-TPase and antiangiogenic effect. To define other structural requirements that confer inhibitory activity to KIN59 and in an attempt to improve the anti-TPase activity, we synthesized several 5′-O-tritylated nucleoside analogs with modifications in the base or small changes in the trityl and/or sugar moiety.

Our studies demonstrate that the anti-TPase activity can be significantly improved by the replacement of the hypoxanthine base by thymine (3.5-fold; KIN6), and by the introduction of chloride on the trityl group (7-fold; TP136). However, removal of 2′-hydroxyl in the sugar part did not significantly affect the inhibitory activity of the compounds, indicating that the 2′-hydroxyl group is not very important for anti-TPase activity. In contrast, anti-TPase activity was completely lost upon substitution of hypoxanthine by an incomplete purine base analog (TP151) or in the absence of the base moiety (TP142). Thus, TPase inhibition may require a ring system such as the heterocyclic purine or pyrimidine base. Moreover, TP124, like KIN59, inhibited TPase in a noncompetitive fashion, indicating that the presence of the trityl group prevents the compound from binding to the thymidine- and phosphate-binding sites in TPase. Therefore, the tritylated compounds most likely bind TPase at a site distant from the phosphate- and dThd-binding sites, obviously at a lipophylic domain of the enzyme. Attempts to reveal the location of the allosteric binding-site of KIN59 at human TPase by crystallography of TPase-KIN59 complexes are currently ongoing.

Also in intact human platelets, which contain high amounts of TPase (Desgranges et al., 1983), KIN59 efficiently inhibited the phosphorolysis of exogenously added dThd, whereas TP142 proved inactive, pointing to the relevance of the cell-free enzyme studies. These findings also indicate that the 5′-O-trityl nucleoside derivatives are taken up by intact (human blood platelet) cells, can exert their anti-TPase activity intracellularly, and must be chemically stable in intact cells. In fact, the compounds proved to be chemically stable in human CEM cell extracts (data not shown). After 24 h of incubation at 37°C, nearly 100% of the original tritylated compounds were recovered. Moreover, any
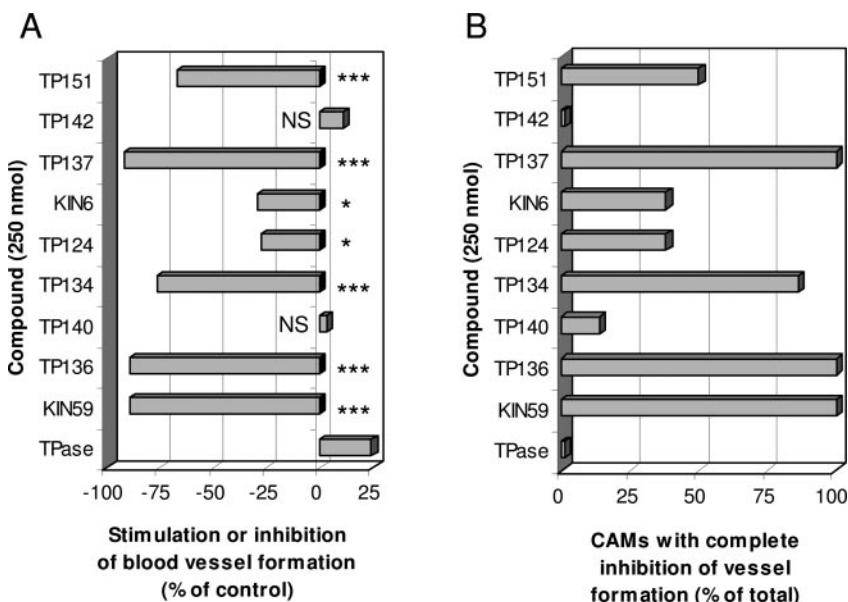
Fig. 4. Effects of 5′-O-tritylated nucleoside analogs on TPase-induced angiogenesis in the CAM assay. At day 9 of incubation, discs containing either TPase or TPase plus test compound were applied onto the CAM. An untreated control disc was positioned on the CAM 1 cm away from the disc containing the test compounds. At day 11, the percentage of stimulation (positive values) or inhibition (negative values) of blood vessel formation, compared with untreated controls, was determined (see *Materials and Methods*). A, change in vascular density of CAM in the presence of TPase plus 250 nmol of various test compounds. B, the percentage of CAMs for which a complete avascular zone was visible after application of TPase plus 250 nmol of various test compounds. *, $p < 0.05$; ***, $p < 0.001$; NS, not significant. Number of blood vessels of untreated controls, 25.7 ± 2.9.

significant conversion of the 5′-O-tritylated thymidine analogs to their free thymine base by TPase or conversion of the 5′-O-tritylated inosine analog KIN59 to its free hypoxanthine base by purified purine nucleoside phosphorylase has never been observed (data not shown). All of these data point to a marked stability of the test compounds in the enzyme assays and intact cell cultures.

Besides an important enzyme in nucleoside metabolism, TPase is also a potent stimulator of endothelial cell migration in vitro and angiogenesis in vivo (Moghaddam et al., 1995). In addition, TPase is overexpressed in several human tumors, and in the majority of these tumors TPase levels cor-

relate well with intratumoral microvessel density and metastasis (Toi et al., 2005). The mechanism by which TPase induces blood vessel development is not completely understood, but the enzymatic activity of TPase was found to be indispensable for angiogenesis stimulation (Miyadera et al., 1995). In particular, one of the products of the TPase reaction [i.e., 2-deoxy-D-ribose, which is obtained by dephosphorylation of 2-deoxy-D-ribose-1-phosphate] was found to produce oxygen radical species, which induce the secretion of oxidative stress-response angiogenic factors, including vascular endothelial growth factor (VEGF), matrix metalloproteinase-1, and interleukin-8 (Brown et al., 2000). Moreover, 2-deoxy-D-ribose has been shown to activate specific integrins, linking TPase-induced endothelial cell migration to intracellular signal transduction pathways (Hotchkiss et al.,
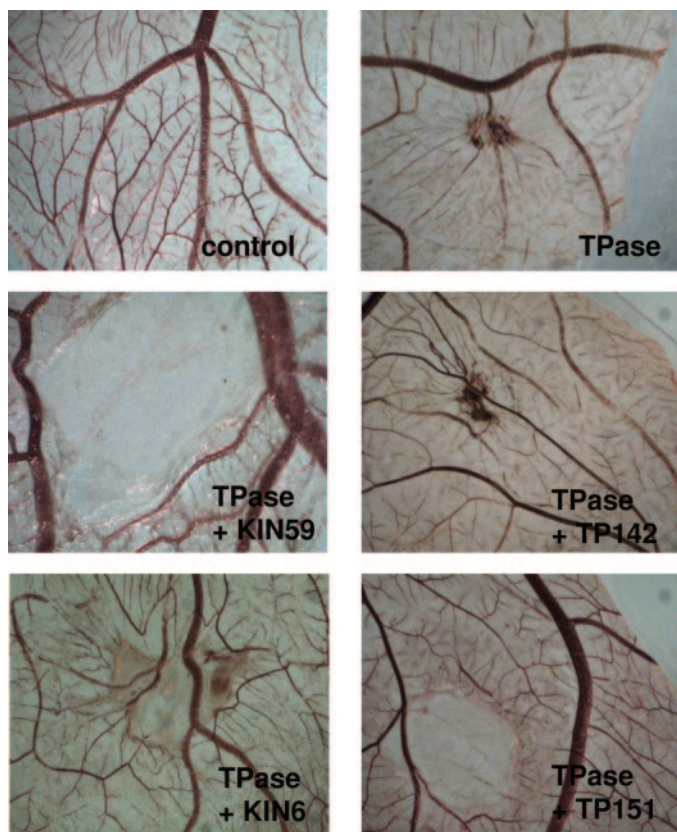

Fig. 5. Effects of KIN59 and derivatives on TPase-induced angiogenesis in the CAM assay. At day 9 of incubation, discs containing either dimethylsulfoxide (DMSO), TPase plus DMSO, or TPase plus 250 nmol KIN59, TP142, KIN6, or TP151 were applied onto the CAM. Two days later, new blood vessels had developed toward the disc containing TPase and TPase plus TP142, whereas a complete absence of capillaries was noted when the disc contained TPase plus KIN59 or TPase plus TP151. In the presence of TPase plus KIN6, some CAMs were characterized by a small avascular spot surrounded by many vessels (see figure), whereas other CAMs showed stimulation of angiogenesis. Original magnification, 15×.

TABLE 2
Effects of KIN59, TP142, KIN6, and TP151 on TPase-induced conversion of dThd to thymine, endothelial cell migration and proliferation in vitro, and TPase-induced angiogenesis in vivo

Values are presented as mean ± S.E.M.

Compound	TPase IC$_{50}$	CAM Assay (250 nmol)[a]	Cell Proliferation IC$_{50}$	Cell Migration
	μM		μM	μM
KIN59	30 ± 8.7	−89% ± 10	78 ± 2	25–100
TP142	≥70	+11% ± 17	39 ± 4	
KIN6	8.6 ± 0.8	−27% ± 16	8 ± 1	25–50
TP151	>75	−67% ± 8	60 ± 1	100

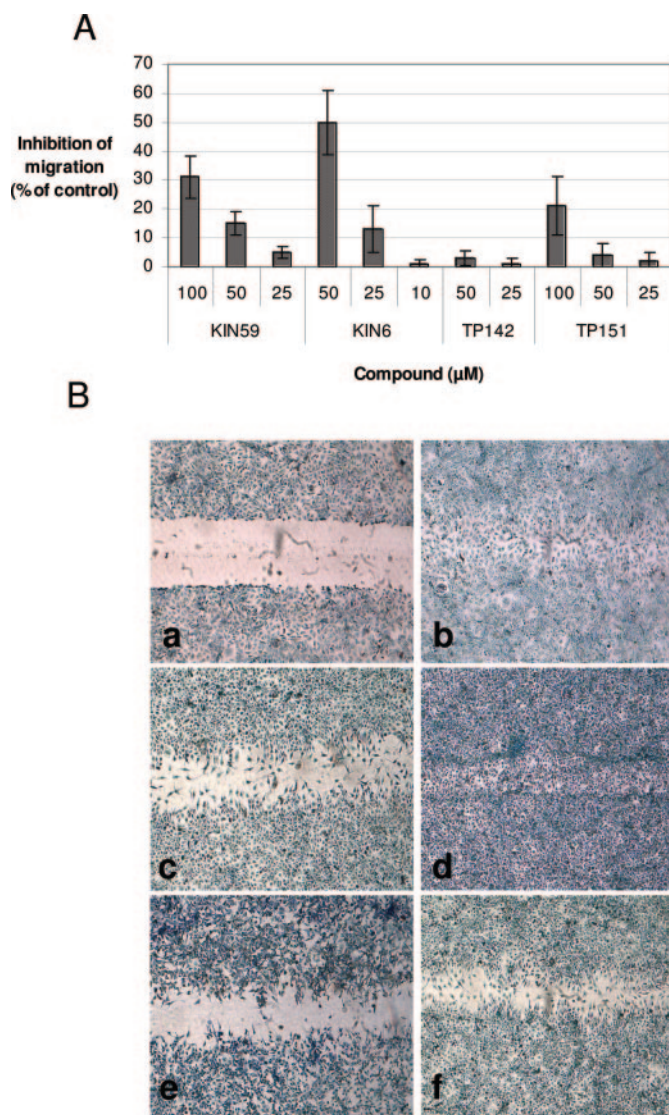[a] Number of blood vessels of untreated controls, 25.7 ± 2.9.

Fig. 6. Effects of KIN59, KIN6, TP142, and TP151 on endothelial wound repair. Confluent MAECs were wounded with a 1.0-mm wide tip and incubated with increasing concentrations of the test compounds. After 16 h, the wounds were photographed (B), and endothelial cells invading the wound were quantified by computerized analysis of the digitalized images (A). B, representative images of control monolayers at 0 (a) and 16 h (b) after wounding. KIN59 (100 μM, c), KIN6 (50 μM, e), and TP151 (100 μM, f) delayed wound closure, whereas TP142 was inactive at 50 μM (d).

2003a,b). The requirement of TPase enzyme activity for angiogenesis stimulation implies that TPase inhibitors should inhibit angiogenesis.

Thus far, we have identified several novel types of TPase inhibitors with concomitant antiangiogenic activity (Balzarini et al., 1998; Liekens et al., 2002). In addition, KIN59, which represents the first compound that inhibits TPase activity without interacting with the substrate-binding sites of the enzyme, displayed a potent antiangiogenic activity in the CAM assay (Liekens et al., 2004). In fact, most of the tritylated nucleoside derivatives with anti-TPase activity completely inhibited TPase-induced angiogenesis and physiological angiogenesis on the CAM, resulting in the appearance of an avascular zone at the site of drug exposure. The potent TPase inhibitors KIN6 and TP124 displayed surprisingly poor effects on blood vessel formation in the CAM assay, whereas TP151, which did not inhibit TPase activity at the highest concentration tested, proved to be a potent antiangiogenic compound in the CAM assay. These apparently contradictory observations could not be explained by toxicity of the test compounds, which could result in an unspecific antiangiogenic activity. Indeed, among the four compounds tested for cytostatic and cytotoxic activity on endothelial cells (KIN59, KIN6, TP151, and TP142), only KIN6 proved relatively toxic and inhibited endothelial cell proliferation at 9-fold lower concentrations than the other compounds. Thus, despite its pronounced antiproliferative activity, KIN6 did not confer a potent antiangiogenic effect in the CAM assay. On the other hand, the marked inhibition of angiogenesis by TP151, which lacks anti-TPase activity in cell-free enzyme assays, could not be attributed to endothelial cell toxicity because of its low cytostatic activity. In addition, a pronounced inhibitory effect on endothelial cell migration could not explain the observed discrepancies. Indeed, both KIN59 and KIN6 dose-dependently inhibited endothelial cell migration, whereas TP151 delayed wound closure only at 100 μM, and TP142 was inactive at nontoxic concentrations. Thus, the TPase inhibitor KIN6 lacked potent activity in the CAM assay but markedly inhibited endothelial cell migration and proliferation in vitro.

The 5'-O-trityl nucleoside derivatives inhibited not only TPase-induced angiogenesis but also the development of vessels that emerged in the absence of exogenously added TPase (i.e., basal angiogenesis in the CAM). Although the expression of TPase during normal CAM development has never been reported, preliminary data indicate that TPase enzymatic activity is present in isolated CAMs at different times of development (S. Liekens, unpublished results). Therefore, inhibition of endogenously produced TPase by 5'-O-trityl nucleoside analogs may result in the abrogation of normal CAM angiogenesis. However, blood vessel formation is also induced by a variety of other angiogenic molecules (Ribatti and Presta, 2002). This implies that KIN59 derivatives may also interfere with other angiogenic target(s) besides TPase. In fact, preliminary data indicate that KIN59 also inhibits basic fibroblast growth factor-induced angiogenesis, but not VEGF-induced angiogenesis in the CAM assay (D. Ribatti, unpublished data), although this finding needs further investigation. A possible interaction of the tritylated nucleoside analogs with angiogenesis stimulators other than TPase may explain the antiangiogenic effect of TP151, which lacked anti-TPase activity.

Tumors have been shown to produce several angiogenic factors, and their presence and concentration may vary during time course and progression of the tumor (Fayette et al., 2005). Thus, the simple concept that tumors may be effectively eradicated by a single antiangiogenic agent has been abandoned (Gasparini et al., 2005). Tumor treatment is likely to benefit from compounds that affect multiple angiogenic pathways. For example, SU-6668, which was developed as an inhibitor of receptor tyrosine kinases involved in VEGF, fibroblast growth factor, and platelet-derived growth factor signaling, is currently being evaluated in clinical trials for the treatment of various human cancers (Laird et al., 2002; Ahmed et al., 2004; Godl et al., 2005). Therefore, the class of 5'-O-trityl nucleoside analogs described in this study represents a new group of molecules that may interfere with one or several angiogenic factors important for tumor progression.

Besides inhibiting angiogenesis, 5'-O-tritylated nucleoside derivatives also caused the degradation of pre-existing vessels at the site of drug administration, whereas the blood vessels at other parts of the CAM remained unaffected. Because we did not observe any toxicity of the compounds to the developing embryo or on cultured endothelial cells in vitro, it is very unlikely that the regression of blood vessels is merely the result of an unspecified toxic effect of the compounds. Other compounds that have been described to cause regression of the pre-existing blood vessels include vascular targeting agents like combretastatin and *trans*-resveratrol (Belleri et al., 2005; Tozer et al., 2005; Vincent et al., 2005). These compounds have been shown to induce the selective apoptosis of angiogenic (i.e., stimulated) endothelial cells and destruction of tumor vasculature (Vincent et al., 2005). In this respect, it may be important to investigate the apoptotic properties of the tritylated nucleoside derivatives.

The antitumor activity of KIN59 and its analogs still has to be established, and we need to investigate whether the compounds are able to induce apoptosis in activated endothelial cells and in tumor vasculature. In addition, it is not yet clear how 5'-O-tritylated nucleoside analogs interact with TPase (or other angiogenesis stimulators) at the molecular level. Further studies are required to reveal the exact mechanism of action of these unusual nucleoside structures, which affect different angiogenic targets. Compounds like KIN59 and its derivatives that combine antiangiogenic and vascular-targeting activities may prove very useful for the design of efficacious anticancer drugs.

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Address correspondence to: Dr. Sandra Liekens, Rega Institute for Medical Research, Minderbroedersstraat 10, B-3000 Leuven, Belgium. E-mail: sandra. liekens@rega.kuleuven.be