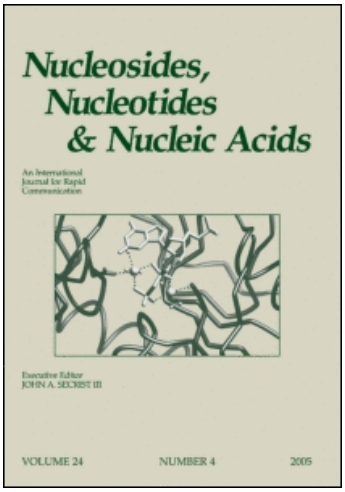

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Thymidine Phosphorylase is Noncompetitively Inhibited by 5'-*O*-Trityl-Inosine (KIN59) and Related Compounds

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THYMIDINE PHOSPHORYLASE IS NONCOMPETITIVELY INHIBITED BY 5′-*O*-TRITYL-INOSINE (KIN59) AND RELATED COMPOUNDS

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□ *We found that 5′-O-trityl-inosine (KIN59) inhibits recombinant bacterial (E. coli) and human thymidine phosphorylase (TPase) with an IC_{50} of 44 μM and 67 μM, respectively. In contrast to previously described TPase inhibitors, KIN59 does not compete with thymidine (dThd) at the pyrimidine nucleoside-binding site or with inorganic phosphate (Pi) at the phosphate-binding site of the enzyme. These findings are strongly suggestive for the presence of an allosteric binding site at the enzyme. TPase is identical to the angiogenic protein platelet-derived endothelial cell growth factor (PD-ECGF). As such, PD-ECGF stimulates angiogenesis in the chick chorioallantoic membrane (CAM) assay. This angiogenic response was completely inhibited by KIN59. Inosine did not inhibit the enzyme or the angiogenic effect of TPase, confirming that the 5′-O-trityl group in KIN59 is essential for the observed effect. Our observations indicate that allosteric sites in TPase may regulate its biological activity.*

Keywords Thymidine phosphorylase; Platelet-derived endothelial cell growth factor (PD-ECGF); Angiogenesis; Cancer; Nucleoside metabolism

INTRODUCTION

Thymidine phosphorylase (TPase) catalyzes the reversible phosphorolysis of thymidine (dThd) and related analogues, to the free (thymine) base

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derivative and 2-deoxy-α-D-ribose-1-phosphate.[1] TPase was found to be identical to an angiogenic protein, designated as platelet-derived endothelial cell growth factor (PD-ECGF) (2) As such, TPase stimulates endothelial cell migration *in vitro* and angiogenesis *in vivo* and plays an important role in tumor growth and metastasis.[2–5] The enzymatic activity of TPase was found to be crucial for its angiogenic effect.[6] However, the mechanism by which TPase induces angiogenesis is still not clear. Recent observations suggest that 2-deoxy-D-ribose, the dephosphorylated product derived from 2-deoxy-α-D-ribose-1-phosphate, stimulates angiogenesis by generating oxygen radical species, which induce the secretion of oxidative stress-responsive angiogenic factors, like vascular endothelial cell growth factor, interleukin-8, and matrix metalloproteinase-1.[7] Moreover, Hotchkiss et al.[8] have shown that TPase and 2-deoxy-D-ribose activate specific integrins, which directly links TPase-induced endothelial cell migration to intracellular signal transduction pathways. However, not all biological actions of TPase could be explained by the release of 2-deoxy-D-ribose.[9]

TPase is overexpressed in many solid tumors and TPase levels correlate well with microvessel density in breast, ovarian, colorectal, endometrial, and esophageal cancers,[4,10] indicating an important role for this enzyme in tumor vascularization. In addition, TPase has been shown to inhibit tumor cell apoptosis.[10] Therefore, there is an anticancer potential for potent and specific TPase inhibitors. Here, we describe the inhibitory activity of the inosine analogue KIN59 (Figure 1) against human and bacterial recombinant TPase. Kinetic analysis revealed that KIN59 does not compete with the phosphate or the nucleoside-binding site of the enzyme, indicating that other, yet unrevealed sites on the TPase enzyme, may be essential to confer certain biological properties to TPase.

MATERIALS AND METHODS

Compound synthesis. 5′-*O*-tritylinosine (KIN59, Figure 1A), was synthesized as described before.[11] ***Purification of recombinant TPase.*** The pMOAL-10T vector containing the human TPase gene (fused to GST) was provided by Prof. R. Bicknell (Oxford, UK). The *E. coli* TPase gene was expressed in *E. coli* as a glutathione-S-transferase (GST) fusion protein. Purification of the proteins was performed as described previously.[12] ***TPase enzyme assays***. The phosphorolysis of thymidine (dThd) by human or *E. coli* GST-TPase was measured by HPLC analysis.[11] ***Chorioallantoic membrane (CAM) assay in fertilized chicken eggs***. Briefly, 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). Next, the eggs were returned to the incubator. At Day 9, the compounds were placed on sterile plastic discs. A solution of cortisone acetate (100 μg/disc, Sigma, St. Louis, MO) was added to all discs

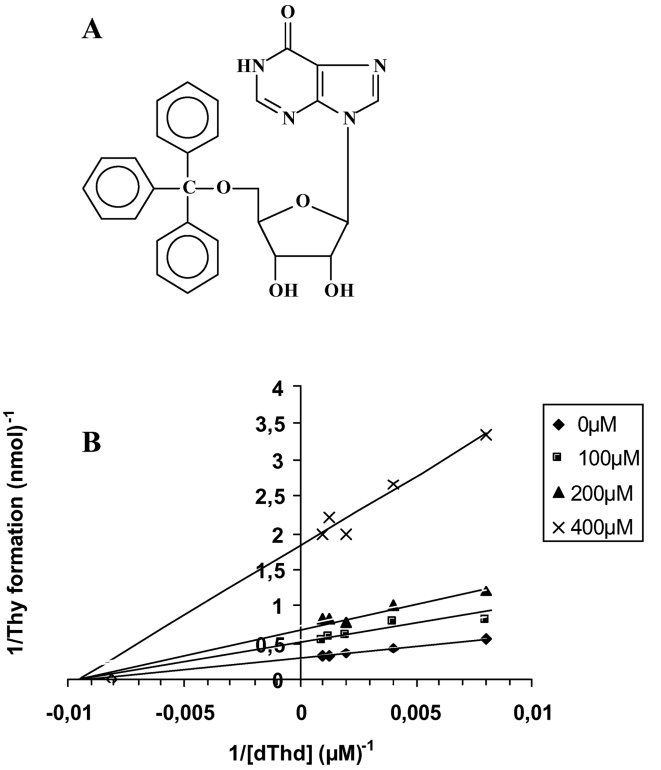

FIGURE 1 Chemical structure of KIN59 (A) and Lineweaver-Burk plot of human TPase inhibition by KIN59 in the presence of variable concentrations of dThd (B).

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 compounds. Next, the eggs were incubated until Day 11 after which the discs were removed and the membranes fixed with 10% buffered formalin (Janssen Chimica, Geel, Belgium). A large area around the discs was cut off and placed on a glass slide. The number of blood vessels was determined microscopically.[11, 12]

RESULTS AND DISCUSSION

The inhibitory activity of KIN59 (Figure 1) against TPase was evaluated in the presence of 100 μM dThd as the natural substrate. KIN59 inhibited the TPase-catalysed conversion of dThd to Thy with a 50% inhibitory concentration (IC_{50}) of 44±3 and 67±20 μM, respectively, when *E. coli* and human TPase were used as the enzyme source. Extensive kinetic studies revealed that KIN59 behaves as a reversible inhibitor of *E. coli* TPase with Ki values of 39 μM against dThd and 146 μM against phosphate, resulting in Ki/Km values of 0.039 and 0.10, respectively. Surprisingly, the compound

inhibited the enzyme reaction in a noncompetitive fashion both with respect to dThd and to phosphate. This means that KIN59 does not bind to either the pyrimidine or phosphate-binding sites of the enzyme. A similar noncompetitive, reversible interaction was observed between human TPase and KIN59 (Figure 1B).

Most TPase inhibitors identified so far are substituted uracil analogues that compete with the pyrimidine-binding site of TPase.[13–16] TPase is known to be highly specific for 2′-deoxyribosyl-derivatives of pyrimidines and this specificity has been explained based on structural features.[17] KIN59 represents a very unusual TPase ligand, being a 5′-*O*-substituted purine riboside instead of a pyrimidine deoxyriboside. The kinetic results obtained for KIN59 against TPase indicate that this compound might interact with a yet unidentified site in the enzyme, different from the pyrimidine and Pi binding sites. In order to evaluate whether the interaction of KIN59 with TPase is sufficient to abrogate the angiogenic activity of TPase, we studied the effect of KIN59 on TPase-induced neovascularization in the chorioallantoic membrane (CAM) assay (Figure 2). We previously showed that 10 units of a commercial batch of pure *E. coli* TPase significantly stimulate the formation of new blood vessels on the CAM of fertilized chicken eggs.[11, 12] After 2 days of exposure to TPase, allantoic vessels developed radially toward the stimulus in a "spoked-wheel" pattern. The effect of TPase on the CAM also has been quantified with an average stimulation of 44%±10 as compared to control CAMs ($p<0.001$). Addition of TPase + 250 nmol of KIN59 resulted in a complete inhibition of TPase-induced angiogenesis (Figure 2, right panel). Moreover, KIN59 not only inhibited the stimulatory effect of TPase, it also prevented the formation of normal CAM vessels in the absence of exogenously added TPase (i.e., normal blood vessel development that occurs between Day 9 and 11) (Figure 2, left panel). Thus, only big veins (that were already present at Day 9 when the compounds were added) were visible after microscopic

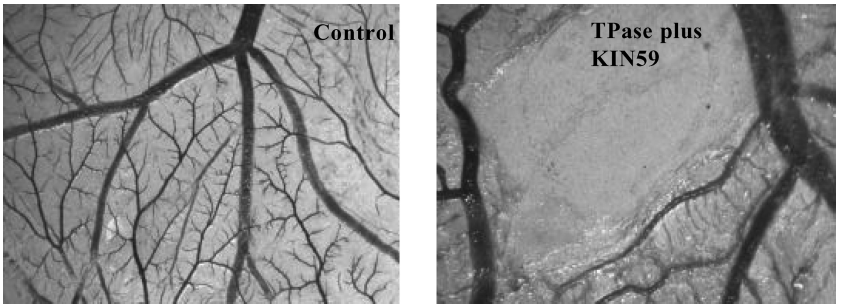

FIGURE 2 Effect of KIN59 on TPase-induced angiogenesis in the chick chorioallantoic membrane (CAM) assay. At Day 9 of incubation, discs containing either DMSO, or TPase plus 250 nmol of KIN59 (in DMSO) were applied onto the CAM. After 2 days, KIN59 has completely inhibited new blood vessel formation.

evaluation of the CAM at Day 11 (i.e., 44% stimulation by TPase in the absence of compound versus 89% inhibition by KIN59 in the presence of TPase). Also at lower doses, KIN59 still significantly inhibited TPase-induced angiogenesis in the CAM. In contrast to KIN59, the 5′-OH free nucleoside inosine did not abrogate the activity of TPase, indicating that the presence of the trityl group is important to afford anti-TPase and anti-angiogenic activity to KIN59. Therefore, we evaluated the effect of several other 5′-*O*-tritylated nucleosides on TPase activity. The trityl group was found to be crucial to maintain anti-TPase activity. However, the purine base of KIN59 could be changed keeping inhibitory activity against TPase.

Our observations indicate that the angiogenic activity of TPase may be influenced through nonsubstrate inhibitors, suggesting that regulatory (allosteric) sites in TPase may play an important role in the angiogenic effect of TPase. Identification of these sites is important to gain more insights in the molecular role of TPase in angiogenic diseases and cancer.

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