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Commentary

# Targeting platelet-derived endothelial cell growth factor/thymidine phosphorylase for cancer therapy

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ABSTRACT

Thymidine phosphorylase (TP) is a key enzyme in the pyrimidine nucleoside salvage pathway, but it also recognizes and inactivates various anti-cancer chemotherapeutic agents. Moreover, TP is identical to platelet-derived endothelial cell growth factor (PD-ECGF), an angiogenic factor with anti-apoptotic properties. Increased expression of PD-ECGF/TP is found in many tumor and stromal cells, and elevated TP levels are associated with aggressive disease and/or poor prognosis. Thus, progression and metastasis of TP-expressing tumors might be abrogated by TP inhibitors that are used as single agents or in combination with (TP-sensitive) nucleoside analogues. On the other hand, increased TP activity in tumors may be exploited for the tumor-specific activation of fluoropyrimidine prodrugs, such as capecitabine. This review will focus on the different biological activities of PD-ECGF/TP and their implications for cancer progression and treatment.



## 1. Introduction

Thymidine phosphorylase (TP) was first described more than 50 years ago [1]. TP catalyses the phosphorolysis of thymidine and 2′-deoxyuridine to their respective base and α-2-deoxy-D-ribose-1-phosphate (Fig. 1). Although the reaction is reversible, the main metabolic function of TP seems to be catabolic, driving the salvage pathway of pyrimidine nucleosides [2]. TP also catalyses the direct deoxyribosyl transfer from deoxyribonucleosides to pyrimidine bases to form a new nucleoside. Moreover, the enzyme recognizes a wide number of 5-substituted 2′-deoxyuridines with antiviral and/or anti-tumoral properties [3–5]. TP may thus inactivate nucleoside analogues, such as 5-trifluorothymidine (TFT), that are being used clinically to treat cancer [5, see further].

In 1987, a ''novel'' protein was extracted from human platelets that induced [$^3$H]thymidine incorporation, endothelial cell chemotaxis *in vitro* and angiogenesis *in vivo* [6,7]. The protein was therefore named platelet-derived endothelial cell growth factor (PD-ECGF). In 1992, it was demonstrated that

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*Abbreviations:* CAM, chick chorioallantoic membrane; 2dR1P, 2-deoxy-D-ribose-1-phosphate; DFUR, 5′-deoxy-5-fluorouridine; FdUrd, 5-fluorodeoxyuridine; 5FU, 5-fluorouracil; HIF-1α, hypoxia-inducible factor-1α; $IC_{50}$, 50% inhibitory concentration; IFN, interferon; IL-8, interleukin-8; MMP, matrix metalloproteinase; MNGIE, mitochondrial neurogastrointestinal encephalomyopathy; PD-ECGF, platelet-derived endothelial cell growth factor; RA, rheumatoid arthritis; TAM, tumor-associated monocytic cell; TFT, trifluorothymidine; TNF-α, tumor necrosis factor-α; TP, thymidine phosphorylase; UP, uridine phosphorylase; VEGF, vascular endothelial growth factor

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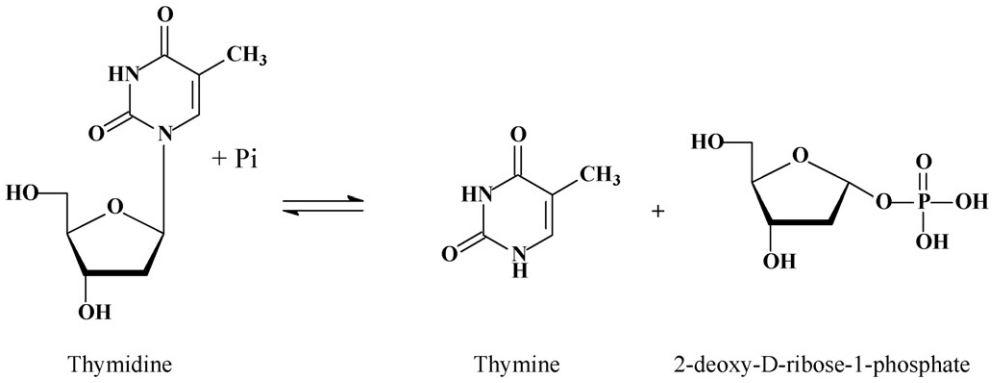


**Fig. 1 – Enzymatic reaction catalysed by TP.**

recombinant human PD-ECGF possesses TP activity [8]. Next, amino acid sequence analysis and gel chromatography revealed that PD-ECGF is identical to TP [9]. Thus, the observed increased uptake of thymidine into cells, in the presence of PD-ECGF, was not related to cell proliferation but a consequence of its TP activity. PD-ECGF hydrolyses serum-derived exogenous thymidine by which a subsequent pulse of radiolabeled thymidine is rapidly taken up by the cells [9].

PD-ECGF/TP was also found to be identical to gliostatin, a protein isolated from human neurofibroma [10]. Gliostatin is a glial growth inhibitory factor with survival-promoting effects on neurons [10]. Based on chemical and biological characteristics, the three proteins are considered identical, although ''PD-ECGF'' and ''TP'' are used interchangeable throughout the literature, whereas the use of ''gliostatin'' is mainly confined to rheumatoid arthritis and neurological research (see further).

## 2. Structure of PD-ECGF/TP

The human PD-ECGF/TP gene is located on chromosome 22q13 and is composed of 10 exons dispersed over a 4.3 kb region [11]. Analyses of the biosynthesis and processing of TP produced by cultured cells revealed that it undergoes limited post-translational modification and is not glycosylated. However, covalent linkage between serine residues of TP and phosphate groups of nucleotides has been observed [12]. The functional significance of this post-translational modification is not clear but it may facilitate secretion of the protein, which does not contain a classical secretion signal [12].

TP is a dimer, consisting of two identical subunits that are non-covalently associated, with a dimeric molecular mass ranging from 90 kD in *Escherichia coli* to 110 kD in mammals. The nucleic acid sequence of TP is highly conserved, i.e. human TP shares 39% sequence identity with *E. coli* TP. Already in 1990, the crystal structure of *E. coli* TP was determined at 2.8 Å resolution [13]. Each subunit is composed of a small α-helical domain that contains the thymidine-binding site and, separated by a cleft, a large α/β domain that contains the phosphate-binding site. Domain movement was suggested to be critical for enzymatic activity by closing the active site cleft, leading to the exclusion of water [reviewed in 14]. The idea of a closed active conformation resulting from rigid body movement of the α and α/β domains was further supported by the crystal structure of pyrimidine nucleoside phosphorylase of *Bacillus stearothermophilus* [14].

In 2004, the crystal structure of human TP was solved in complex with the substrate analogue TPI [15]. The complex was found to be in a closed (active) conformation and TPI appeared to mimic the substrate transition state. More recently, another structure of human TP was determined [16]. This new structure, in contrast to the previous one, corresponds to a non-trypsinised protein (from residue 34–479). Thus, structural information on human TP becomes increasingly available and will be of value for the design of new inhibitors [16].

## 3. Angiogenic activity of PD-ECGF/TP

Angiogenesis is the formation of new blood vessels from the pre-existing vasculature. It is a complex multi-step process that involves the tightly regulated action of a large number of angiogenesis mediators (i.e. extracellular matrix components, growth factors, integrins, cytokines, enzymes) with either inhibitory or stimulatory function. Unregulated angiogenesis contributes to several angiogenic diseases, such as rheumatoid arthritis, atherosclerosis and diabetic retinopathy, and is indispensable for solid tumor growth and metastasis [reviewed in 17,18]. Recently, the first anti-angiogenic agent Avastin, a humanized monoclonal antibody against vascular endothelial growth factor (VEGF), has been approved (in combination with fluorouracil-based drug regimens) for the treatment of metastatic colorectal cancer [19].

TP stimulates endothelial cell migration and tube formation *in vitro* [6,20,21], and angiogenesis in different *in vivo* models, such as the chick chorioallantoic membrane (CAM) assay, subcutaneously implanted sponges in mice and rats, and a freeze-injured skin graft model in mice [20,22,23]. Selective overexpression of TP in MCF-7 breast cancer cells did not affect their growth *in vitro* but resulted in more vascularized and faster growing tumors *in vivo* [22]. Moreover, TP expression increased the metastatic potential of experimental and human tumors (see further). In human melanoma xenografts that express multiple angiogenic factors, lung colonization and spontaneous metastasis were inhibited by treatment with neutralizing antibodies against TP, indicating that TP may promote melanoma metastasis [24].

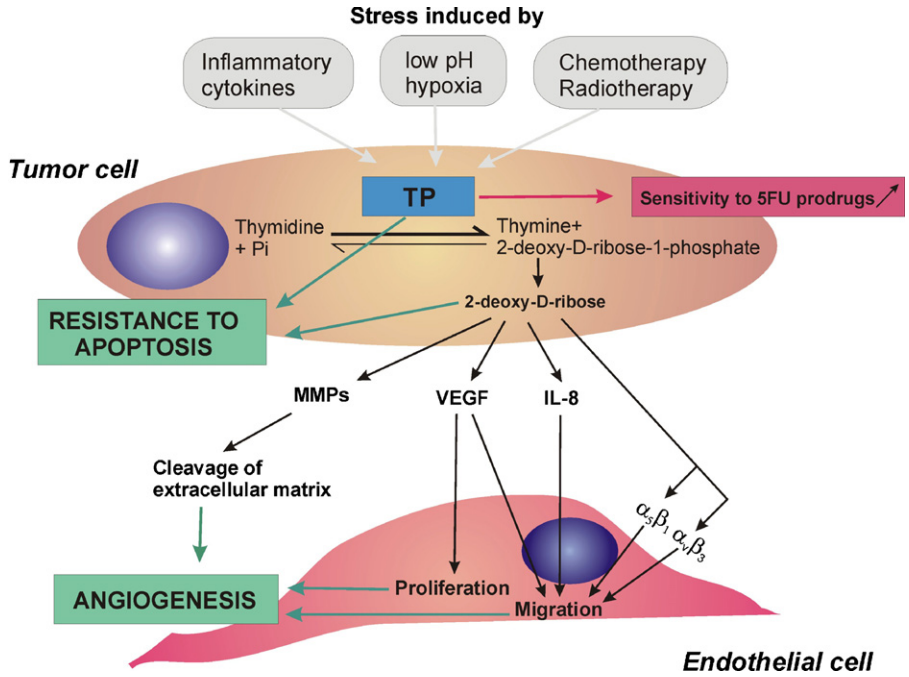


**Fig. 2 – Schematic representation of the role of TP and 2-deoxy-D-ribose in cancer. Various conditions of cellular stress, such as low pH, hypoxia, cytokines, radio- and chemotherapy, may induce the expression of thymidine phosphorylase (TP) in tumors. TP catalyses the reversible phosphorolysis of thymidine to thymine and 2-deoxy-D-ribose-1-phosphate, which is converted to 2-deoxy-D-ribose. TP and 2-deoxy-D-ribose promote tumor growth by two distinct mechanisms: (1) by promoting angiogenesis and (2) by inhibiting apoptosis. (1) 2-Deoxy-D-ribose may generate oxygen radicals that increase the production of oxidative stress-response angiogenic factors, such as IL-8, VEGF and MMPs. It can also induce endothelial cell migration via direct activation of the integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$. (2) Tumors overexpressing TP are protected from apoptosis induced by Fas, hypoxia, DNA damage and microtubuli-destabilizing agents. In contrast to its tumor-promoting properties, the enzymatic activity of TP is required for the activation of 5FU prodrugs.**

Most angiogenic factors bind to high-affinity tyrosine kinase receptors on the cell surface, resulting in the activation of intracellular signal transduction pathways, and subsequent endothelial cell responses. In contrast, no receptor for TP has been identified. In fact, TP does not contain a secretion signal and is predominantly found intracellulary although some tumor cells, such as A431 and MKN74, release the protein into the cell culture medium [25]. Several experimental data indicate that the enzymatic activity of TP is indispensable for its angiogenic effect: (i) the dephosphorylated enzymatic product, 2-deoxy-D-ribose, also induced angiogenesis, (ii) the angiogenic effect of TP was abolished by the competitive TP inhibitor 6-amino-5-chlorouracil, (iii) by neutralizing antibodies and (iv) by mutagenesis of the enzyme's active site residues [20,22,26]. Although metabolites of thymine (i.e. β-amino-iso-butyric acid) have been reported to possess angiogenic activity *in vitro* [21], the main enzymatic product responsible for the angiogenic activity of TP is assumed to be 2-deoxy-D-ribose, which is obtained by rapid dephosphorylation of 2-deoxy-D-ribose-1-phosphate (2dR1P) [20,27]. In particular, Hotchkiss et al. [28] have demonstrated that TP-induced endothelial cell migration requires the conversion of 2dR1P to 2-deoxy-D-ribose. Moreover, the stimulatory effect of TP-expressing tumor cells or monocytes on endothelial cell migration could not be abrogated with a neutralizing antibody against TP, even though the antibody completely blocked migration induced by purified TP. This implies that the intracellular catabolism of thymidine and subsequent extracellular release of 2-deoxy-D-ribose, is required, and sufficient, for angiogenesis stimulation [28].

The mechanism by which TP, and 2-deoxy-D-ribose, induce neovascularization is still not completely understood. Addition of thymidine to a TP-overexpressing carcinoma cell line resulted in increased cellular levels of heme oxygenase 1 (HO-1), a marker for oxidative stress [27]. Up-regulation of HO-1 was inhibited by excess thymine, which acts as a scavenger for 2dR1P. Brown et al. [27] hypothesized that 2-deoxy-D-ribose is a strongly reducing sugar, which may generate oxygen radicals that induce the secretion of oxidative stress-response angiogenic factors, including VEGF, matrix metalloproteinase-1 (MMP-1) and interleukin-8 (IL-8) (Fig. 2). TP activity was also shown to increase the levels of hypoxia-inducible factor (HIF)-1α during *in vitro* hypoxia in RT112 cells, and TP and HIF-1α acted in concert to induce VEGF secretion [29]. In addition, TP was found to increase VEGF mRNA levels and protein secretion in human fibroblast-like synoviocytes, indicating that TP may also regulate VEGF expression in inflamed joints [30].

TP was shown to induce the expression of several matrix metalloproteinases (MMPs), which may provide an explanation for the increased metastatic potential of experimental and human tumors that express the enzyme (Fig. 2). Indeed, MMP-induced degradation of the extracellular matrix surrounding the blood vessels greatly facilitates the escape of

tumor cells into the circulation. TP-overexpressing KK47 bladder and PC prostate cancer cells had higher levels of MMP-7 and MMP-9 than their mock-transfected counterparts under hypoxia [31]. In human cervical carcinoma cell lines, TP mRNA and protein levels correlated well with MMP-2 expression, and with cell migration and invasion *in vitro* [32]. In addition, TP present in rheumatoid arthritis (RA) synoviocytes was found to stimulate the expression of MMP-1 and MMP-3 mRNA, indicating that the extensive joint destruction, seen in RA may be mediated via TP-induced MMPs [33].

## 4. PD-ECGF/TP expression in physiological angiogenesis

Under physiological conditions, TP is highly expressed in the blood. The highest TP activity was detected in platelets suggesting a role for this enzyme in wound healing [34]. TP activity was also detected in peripheral blood mononuclear cells and in the plasma, probably as a result of cell turnover, or originating from damaged platelets. In addition, high levels of TP have been observed in placenta [35], where it may contribute to decidualization of the endometrium [36]. In normal proliferative endometrium, TP shows a specific pattern of distribution that is dependent on the phase of the menstrual cycle, i.e. TP expression is inversely correlated with oestradiol concentrations [37]. In contrast, in endometrial hyperplasia the enzyme is randomly distributed and lacks an orderly pattern [38]. The TP promoter contains half-palindromic oestrogen response elements, and TP expression was induced by the combination of progesterone and transforming growth factor-β1 [39]. In early pregnancy, high concentrations of TP and VEGF in the trophoblast indicate an important role for both angiogenic factors throughout gestation [40]. TP thus seems to be particularly important for processes that require remodeling of the existing vasculature, rather than for developmental angiogenesis.

To assess the physiological functions of TP, mice deficient in both TP and uridine phosphorylase (UP) were generated [41]. Double knock-outs ($TP^{-/-}$ $UP^{-/-}$) were constructed to completely inhibit TP activity as mouse UP can also cleave thymidine (in contrast to human UP, which only cleaves uridine). Targeted deletion of the TP and UP genes in mice did not cause developmental defects. Moreover, $TP^{-/-}$ $UP^{-/-}$ mice appeared healthy and survived up to 16 months. TP deficiency was hypothesized to cause increased plasma thymidine levels, leading to mitochondrial DNA alterations and mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) [42,see further]. However, although thymidine levels were increased in $TP^{-/-}$ $UP^{-/-}$ mice, no mitochondrial alterations or pathological signs of MNGIE could be observed [41].

## 5. Role of PD-ECGF/TP in cancer progression and metastasis

### 5.1. *TP as a marker of tumor progression and prognosis*

Western blot and histological analyses of a variety of human tumors showed increased expression of TP in the tumors, compared to the corresponding non-neoplastic regions of the same organs. Elevated TP levels were associated with increased microvessel density in colorectal, gastric, esophageal, pancreatic, renal, endometrial, breast, ovarian, cervical, prostate, non-small cell lung cancer and glioblastoma, pointing to an important role for TP in tumor vascularization [43–49, reviewed in 50]. Moreover, TP expression correlated well with tumor grade, metastasis and shorter patient survival in colorectal, pancreatic, renal, ovarian, cervical, bladder and non-small cell lung cancer [43,45–47,51, reviewed in 50]. Besides tumor cells, also endothelial cells, lymphocytes, fibroblasts and in particular tumor-infiltrating macrophages were found to express TP. In fact, tumor-associated monocytic cells (TAMs) are major components of tumor stroma that generate various mediators. As such, TAMs may function in diverse manners, i.e. anti-tumor and pro-tumor. TP has been proposed to be a marker of pro-tumor monocytes in breast cancer [52].

Also the concentration of TP in the serum of cancer patients may have prognostic value. TP in serum of patients with uterine cervical cancers was significantly correlated with clinical stage, tumor size and lymph node metastasis. In addition, patients with high TP levels had extremely poor prognosis [53]. Moreover, high serum TP levels in patients with esophageal squamous cell carcinoma were found to be associated with depth of tumor invasion and poor response to treatment [54]. The serum TP level may also represent a novel marker to predict occurrence of hematogenous metastasis in patients with resectable colorectal cancer [55].

Although many studies have shown a positive correlation between TP and tumor progression, some reports indicate otherwise. Discrepancies between different studies may, at least in part, be due to differences in the histological type of cancer, number of patients examined, antibody used for immunohistochemistry, and assays to measure TP. In endometrial, breast and prostate cancer, TP levels in the tumor cells did not correlate with any clinicopathologic factor [56,57]. However, in these tumors, TP expression in stromal cells, in particular activated macrophages, was associated with angiogenesis, tumor grade and prognosis. In pancreatic cancer, the frequency of hepatic metastasis was significantly correlated with TP expression in tumor cells, whereas local recurrence was significantly higher in patients with positive staining for TP in the stromal cells [58].

### 5.2. *Mechanisms of TP up-regulation in cancer*

Besides its constitutive expression by tumor cells and TAMs, TP may be up-regulated in tumors by various cytokines (Fig. 2). Tumor necrosis factor-alpha (TNF-α) was shown to increase TP mRNA levels and TP activity in human colon carcinoma cells via the activation of SP1 transcription factors. In the monocytic cell line THP-1, TNF-α-mediated up-regulation of TP expression involved TNF-α receptor 2 and NFκB [59,60]. TP mRNA levels are extremely low in most cell lines *in vitro*. However, interferon (IFN)-α/β caused a rapid and transient increase of TP expression in HT29 colon cancer cells by stimulation of a putative IFN-stimulated response element (ISRE) in the TP promoter [61]. IFN-γ stimulated the expression of TP in human monocytic U937 cells by increasing the binding

of signal transducer and activator of transcription (STAT) 1 to a gamma-activated sequence-like element in the TP promoter [62]. Besides regulating transcription of TP, interferons also cause prolonged stabilization of TP mRNA, which probably involves a pyrimidine-rich sequence at the 3′ end of the TP mRNA [61].

Current cancer treatment protocols, such as X-ray irradiation and various chemotherapeutic agents, including paclitaxel, docetaxel, cyclophosphamide and mitomycin C may induce TP expression in the tumors via the up-regulation of TNF-α, IFN-γ or interleukin (IL)-10 and IL-1α [63,64]. Conversely, prolonged exposure of human monocytic/macrophage THP1 and U937 cells to the anti-inflammatory drug and NFκB inhibitor sulfazaline resulted in the downregulation of TP and IL-8 (mRNA, protein and activity), along with elimination of their induction by TNF-α and IFN-γ [65]. Thus, inhibition of the NFκB pathway provokes a marked downregulation of macrophage-related angiogenic factors. Also, blockade of paclitaxel-induced TP expression by TP antisense transfection accelerated paclitaxel-induced apoptosis of human prostate cancer cells [66].

Finally, the tumor microenvironment, in particular hypoxia and low pH, may cause an increase in TP protein level and enzymatic activity [67]. This can explain why increased expression of TP was detected in those parts of the tumor that are proximal to necrotic areas, or after occlusion of the tumor vascular supply [67].

### 5.3. *Cooperative action of TP and VEGF*

TP and VEGF are frequently co-expressed in highly vascularized human tumors, including breast, lung, head and neck, colorectal, cervical and endometrial carcinomas and gliomas [44,48,51,68,69]. Co-expression of both factors may, at least in part, be explained by the presence of SP1 transcription factor binding-sites in the TP and VEGF promoter, indicating similar transcriptional regulation of the *TP* and *VEGF* genes [70]. Moreover, Hotchkiss et al. [71] showed that the combined effects of TP and VEGF on migration of human umbilical vein endothelial cells (HUVEC) are additive up to the maximum of each individual agent, suggesting that both proteins may use similar signal transduction pathways. However, TP-induced endothelial cell migration and phosphorylation of focal adhesion kinase were mediated by integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$, whereas VEGF-induced migration only involved $\alpha_v\beta_3$ [71]. Thus, there may be some overlap in the signal transduction pathways of VEGF and TP in HUVEC, but most likely both factors cooperatively stimulate angiogenesis.

Interestingly, in stage I lung adenocarcinoma, TP induction was particularly evident in the tumor stroma, whereas VEGF was highly expressed in the tumor cells [72]. In endometrial carcinoma, HIF-1α expression was associated with up-regulation of the VEGF pathway, whereas HIF-2α expression correlated with TP expression [73]. Thus, TP and VEGF, two potent angiogenic factors with different characteristics with respect to endothelial cell stimulation, may promote angiogenesis cooperatively, resulting in a more aggressive tumor phenotype. Indeed, patients with stage I lung adenocarcinoma that were stromal cell-TP-positive and tumor cell-VEGF-positive had a significantly worse prognosis than patients in the stromal cell-TP-negative tumor group or the tumor cell-VEGF-negative group [72].

### 5.4. *TP-mediated inhibition of tumor cell apoptosis*

TP was also shown to affect tumor growth, independent of its angiogenic activity. Transfection of KB (human epidermoid carcinoma) cells with TP cDNA (KB/TP) rendered them resistant to hypoxia-induced apoptosis [reviewed in 74]. 2-Deoxy-D-ribose and thymine could partially prevent hypoxia-induced apoptosis in KB/TP cells, and this effect was abrogated by the stereoisomer 2-deoxy-L-ribose. 2-Deoxy-D-ribose also prevented hypoxia-induced HIF-1α expression, downregulation of Bcl-2 and Bcl-$X_L$, mitochondrial cytochrome c release, loss of mitochondrial transmembrane potential and caspase-3 activation in human myeloid leukemia HL60 cells, indicating that TP protects cells from hypoxia-induced death via its enzymatic activity [74].

In contrast, TP suppression of Fas-induced apoptosis (i.e. caspase-8 activation and downstream effects) in KB/TP cells did not require its enzymatic activity, as treatment of KB/TP cells with a TP inhibitor, or treatment of control transfectant KB cells with 2-deoxy-D-ribose did not affect cell survival [74 and references herein]. In addition, both TP and a mutant TP devoid of enzymatic activity (L148R) inhibited apoptosis, induced by cisplatin in transfected human leukemia Jurkat cells [74]. Jurkat/TP cells were also more resistant to apoptosis induced by various microtubule-interfering agents, including vincristine, vinblastine, paclitaxel and 2-methoxyestradiol. The enzymatic activity of TP was not required for this protective effect, which was mediated by the inhibition of Bcl-2 phosphorylation and suppression of FasL [75]. Finally, inhibition of DNA-damage-induced apoptosis in TP-transfected U937 cells was independent of TP enzymatic activity and involved, at least in part, the phosphoinositol-3-kinase (PI3K)/Akt pathway [76]. These data indicate that TP may contribute to the progression of TP-expressing tumors by inhibiting apoptosis, although the mechanism seems to depend on the micro-environmental and cellular conditions.

## 6. PD-ECGF/TP in inflammatory and/or angiogenic diseases

A great number of studies have implicated TP in various angiogenic and/or inflammatory diseases. Elevated TP activity (20-fold) was detected in psoriatic lesions, relative to non-lesional skin, where it may provide the thymidine necessary for keratinocyte proliferation [77]. TP has also been involved in the pathology of gastric ulcers [78], and TP immunoreactivity was correlated with the number of lesion microvessels and mast cells in atherosclerotic plaques, implying a role for TP in atherosclerotic vascular remodeling [79]. In addition, strong staining for TP was observed in sections of colonic mucosa of inflammatory bowel disease, whereas TP expression was absent in normal mucosa [80].

High concentrations of TP have also been detected in synovial fluid and serum of rheumatoid arthritis (RA) patients [81,82]. Moreover, the serum TP level, mostly derived from cytokine-stimulated synoviocytes, was a useful clinical marker of RA [83]. In cultured rheumatoid fibroblast-like

synoviocytes, the expression of TP mRNA was significantly increased by stimulation with IL-1β, and reduced by treatment with anti-rheumatic drugs like aurothioglucose and dexamethasone, indicating that the anti-rheumatic activity of these compounds may, at least in part, be mediated by their suppression of TP expression [84]. The extensive joint destruction in RA may be mediated by MMPs, which are induced by TP in synoviocytes and chondrocytes [33,85]. TP was also found to induce VEGF expression and secretion in human fibroblast-like synoviocytes. These data suggest that TP stimulates angiogenesis in RA indirectly, via the action of MMPs and VEGF [86]. Conversely, intra-articular injection of purified recombinant TP in rabbits resulted in the development of diffuse synovitis resembling RA [87].

The above-mentioned diseases are characterized by extensive angiogenesis. However, also the inability to build-up an adequate angiogenic response may lead to vascular disorders, such as myocardial and peripheral ischemia. In these cases exogenous angiogenic factors may be administered to reduce local hypoxia in areas of hypovascularization. A combination plasmid phVEGF165-TP·MB encoding both the VEGF and TP gene has been proposed as a strategy to improve angiogenic gene therapy [88], although no data have been provided to support this hypothesis. However, administration of a plasmid vector containing human TP proved effective in the treatment of myocardial ischemia in dogs [89].

## 7. Involvement of PD-ECGF/TP in MNGIE

Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) is an autosomal recessive human disease associated with multiple deletions of skeletal muscle mitochondrial DNA. The origin of the mutation was located on chromosome 22q13.32, where the gene for TP is located. TP activity in leukocytes of MNGIE patients was less than 5% of controls, suggesting that loss-of-function mutations in TP may cause the disease [90]. TP deficiency leads to increased circulating levels of thymidine and deoxyuridine, causing alterations of nucleoside metabolism, which can induce deletions and point mutations in mitochondrial DNA [42]. It has thus been proposed that MNGIE patients may benefit from therapies that reduce thymidine levels.

In contradiction with these data, a recent study by Kumagai et al. [91] suggests that TP gene mutation is not the primary cause of MNGIE, as the TP gene mutation was also found in unrelated individuals with normal TP activity. Moreover, mice deficient in TP activity do not present mitochondrial alterations or pathological signs of MNGIE, indicating that loss-of-function mutations in the TP gene may not be sufficient to cause the disease [41]. Examination of the genes located on chromosome 22q13.32 revealed that *TP* overlaps with the *SCO2* gene, which may thus contribute to MNGIE [41]. Additional studies are required to clarify the role of TP in MNGIE.

## 8. PD-ECGF/TP inhibitors

In view of the important role of TP in cancer progression and metastasis, it has been proposed that TP inhibitors may significantly improve future cancer therapy, i.e. TP inhibitors could inhibit TP-mediated biological functions, such as angiogenesis stimulation and apoptosis inhibition, but they may also improve the anti-tumor activity of various 2′-deoxypyrimidine nucleoside derivatives that are degraded by TP [92]. At this moment, there are no TP inhibitors approved for clinical use, although several drugs have been tested preclinically and clinically [for an extensive review, see Pérez-Pérez et al. 14].

### 8.1. *Pyrimidine analogues*

For more than 30 years, the reference compounds for TP inhibition were 6-aminothymine and derivatives of 6-aminouracil, such as 6-amino-5-chlorouracil and 6-amino-5-bromouracil (Fig. 3) with $IC_{50}$ values in the micromolar range [93]. As the stimulatory role of TP in angiogenesis and cancer became evident, interest was raised in the synthesis of novel TP inhibitors. These efforts resulted in compounds with increased solubility compared to the reference compounds, and improved inhibitory activity against TP [reviewed in Pérez-Pérez et al. 14].

In particular, 5-chloro-6-[1-(2-iminopyrrolidinyl)methyl] uracil hydrochloride (TPI, Fig. 3) proved to be 1000-fold more active than previously described TP inhibitors, with an $IC_{50}$ value of 35 nM against human TP [92]. TPI suppressed migration and basement membrane invasion of TP-overexpressing KB cells *in vitro* and the angiogenic activity of these cells *in vivo* [94]. In addition, TPI was shown to decrease the growth rate, increase the apoptotic index, and suppress liver metastases of KB/TP cells xenografted into nude mice [94,95].

Based on these data, TPI was considered for combination therapy with trifluorothymidine (TFT), an anti-tumoral agent that is rapidly inactivated by TP. TAS-102, an orally administrable combination of TFT and TPI (at a molar ratio of 1:0.5) is currently being evaluated in phase I studies for patients with various solid tumors [96]. Preclinical data showed that this combination suppresses TP-induced angiogenesis, enhances the anti-tumor efficacy, and decreases the toxicity of TFT in nude mice. Moreover, TAS-102 inhibited liver metastasis in a mouse model of human colorectal cancer and proved to be equally effective against parental and 5-fluorouracil (5FU)-resistant xenografts [97]. As 5FU is widely used in the treatment of solid tumors, and resistance of certain tumors to 5FU therapy presents a major clinical problem, TAS-102 offers interesting perspectives for clinical use.

In order to selectively deliver TP inhibitors at the tumor site where TP is highly expressed, several prodrugs have been designed and synthesized. These compounds rely upon the hypoxic conditions in the tumor microenvironment for their bioreductive activation, in particular the high concentration of xanthine oxidase in many tumors. Thus, Reigan et al. [98] designed 5-halo-6-[(2′-nitroimidazol-1′-yl)methyl]uracil prodrugs that are reduced by xanthine oxidase to the 2′-amino analogues that are potent TP inhibitors [reviewed in 14].

### 8.2. *Purine analogues*

Molecular modeling was used to design the first purine derivatives with inhibitory activity against TP. 7-Deazaxanthine (7DX, Fig. 3) may be regarded as a pyrimidine (6-aminothymine) at which a second ring (on the pyrimidine base) has been added

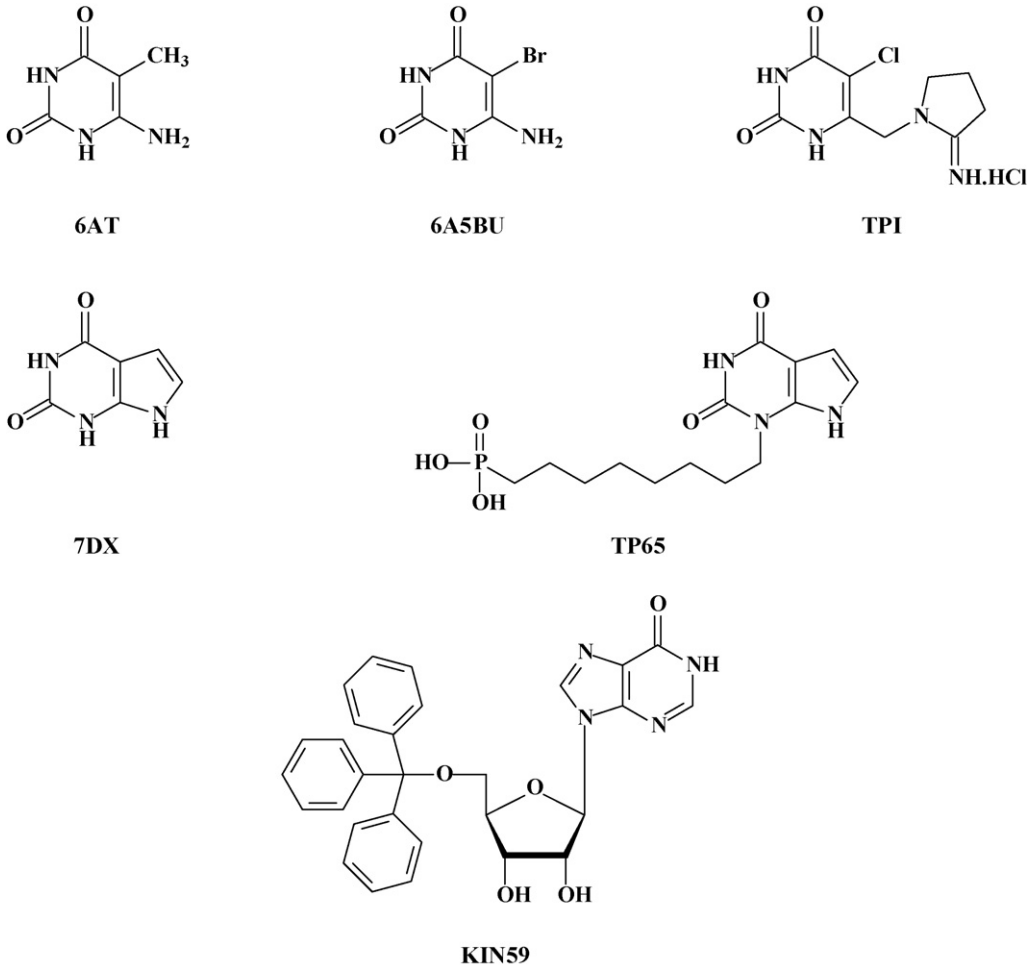


**Fig. 3 – Chemical structure of TP inhibitors.**

in order to create extra stabilizing interactions [reviewed in 14]. Its inhibitory activity was comparable to that of the reference drug 6-aminothymine.

The crystal structure of *E. coli* TP was also used for the design of multisubstrate inhibitors. In its open, inactive conformation, the distance between the thymine- and phosphate-binding site of *E. coli* TP is approximately 8–10 Å. Based on these coordinates, compounds were designed that contain a thymine base, interacting at the nucleoside-binding site, a spacer of six to nine atoms, and a phosphonate moiety that would bind to the phosphate-binding site. Replacement of thymine in such molecule by 7-DX led to TP65 (Fig. 3). This compound inhibited *E. coli* and human TP with $IC_{50}$ values in the micromolar range. Enzyme kinetic experiments showed that TP65 is able to interact concomitantly with the two different substrate-binding sites, thereby immobilizing the enzyme in an open, inactive conformation [14,99]. TP65 also inhibited TP-induced formation of microvascular sprouts from endothelial cell aggregates in a 3D fibrin gel, and TP-induced angiogenesis in the CAM assay [23].

We recently described the inhibitory activity of the purine riboside derivative KIN59 (5′-*O*-tritylinosine; Fig. 3) against TP enzymatic activity ($IC_{50}$ value of 30 μM) and TP-induced angiogenesis in the CAM assay [100,101]. In contrast to previously described TP inhibitors, KIN59 does not compete with the nucleoside- or phosphate-binding site of the enzyme. These data suggest that the enzymatic and angiogenic activity of TP is not solely directed through its functional substrate-binding sites and that a, yet unidentified, allosteric site in TP may play an important role in its biological properties [100].

### 8.3. *2-Deoxy-L-ribose*

A number of studies have shown that different biological effects of 2-deoxy-D-ribose on angiogenesis are suppressed by its stereoisomer 2-deoxy-L-ribose. Indeed, 2-deoxy-L-ribose suppressed 2-deoxy-D-ribose-induced endothelial cell migration in a Boyden chamber, tube formation in collagen gel, and invasion of KB cells overexpressing TP (KB/TP) into matrigel [74,102]. *In vivo*, 2-deoxy-L-ribose inhibited angiogenesis induced by implantation of (i) recombinant TP in the rat cornea, and (ii) KB/TP cells in a mouse dorsal air sac assay [reviewed in 74]. 2-Deoxy-L-ribose also significantly reduced the growth of KB/TP cells xenografted into nude mice, and experimental liver metastasis, induced by these cells [74,102]. The stimulatory effect of TP and 2-deoxy-D-ribose on VEGF and IL-8 expression and secretion from KB/TP cells were also significantly reduced by 2-deoxy-L-ribose, as well as the

expression of MMP-9 [102,103]. In contrast to TP inhibitors, 2-deoxy-L-ribose abrogates the biological activities of TP without affecting its enzymatic activity. This may offer advantages for its use in combination therapy with 5FU prodrugs that require activation by TP (see below).

## 9. TP-mediated activation of anti-cancer prodrugs

### 9.1. *Interaction of TP with fluorinated pyrimidines*

5FU is an antimetabolite, which is part of many cytotoxic drug regimens for the treatment of various malignant diseases. In particular, the compound has been used for more than a decade as first line treatment for colorectal carcinoma [reviewed in 104, Fig. 4]. Cytotoxicity of 5FU mainly results from incorporation of the drug into replicating RNA, and thymidine depletion following thymidylate synthase inhibition. This requires conversion of 5FU to, respectively, FUTP and FdUMP. Only in the presence of sufficient amounts of the co-substrate (2dR1P) for TP, i.e. by adding deoxyribose-1-P donors, TP can convert 5FU to FdUrd. The latter nucleoside analogue is readily phosphorylated by thymidine kinase to FdUMP, which will then inhibit *de novo* thymidylate synthesis [104, Fig. 5]. Clinical efficacy of 5FU is limited by its rapid degradation (by dihydropyrimidine dehydrogenase) resulting in a plasma half-life as short as 6–20 min. In order to maintain effective blood concentrations, and because of its low gastrointestinal absorption, the compound has to be administered intravenously. Therefore, significant efforts have been performed to obtain efficient prodrugs of 5FU.

Doxifluridine (5′-deoxy-5-fluorouridine, DFUR, Fig. 4) is an oral prodrug of 5FU that requires TP for its conversion to 5FU (Fig. 5). As TP is highly expressed in tumors, but also in the gastrointestinal tract, DFUR therapy resulted in a dose-limiting toxicity (diarrhea). Capecitabine (*N*-4-pentyloxycarbonyl-5′-deoxy-5-fluorocytidine, Xeloda, Fig. 4) was designed to bypass this toxicity by selectively delivering 5FU to cancer cells [105]. Oral capecitabine passes through the intestinal tract in its intact form and is converted to 5′-deoxy-5-fluorocytidine by carboxylesterase in the liver. It is subsequently deaminated to DFUR by cytidine deaminase, which is found at high concentrations in the liver and various types of tumors. Finally, DFUR is converted to 5FU by TP, which is up-regulated in tumors compared to normal tissue [106]. Clinical trials with single-agent capecitabine showed improved tolerability and comparable efficacy to intravenous 5FU/leucovorin therapy in patients with metastatic colorectal cancer [reviewed in 107, 108].

### 9.2. *Modulation of fluoropyrimidine sensitivity*

TP represents the rate-limiting enzyme in the activation of DFUR and capecitabine, suggesting that sensitivity of tumor cells to these prodrugs might be enhanced by increasing TP expression. Indeed, transfection of different human tumor cells, including MCF-7 breast carcinoma, PC-9 human lung adenocarcinoma, human KB epidermal carcinoma and Colo320 colon and WiDR lung cancer cells with cDNA of human TP resulted in increased sensitivity of the cells to

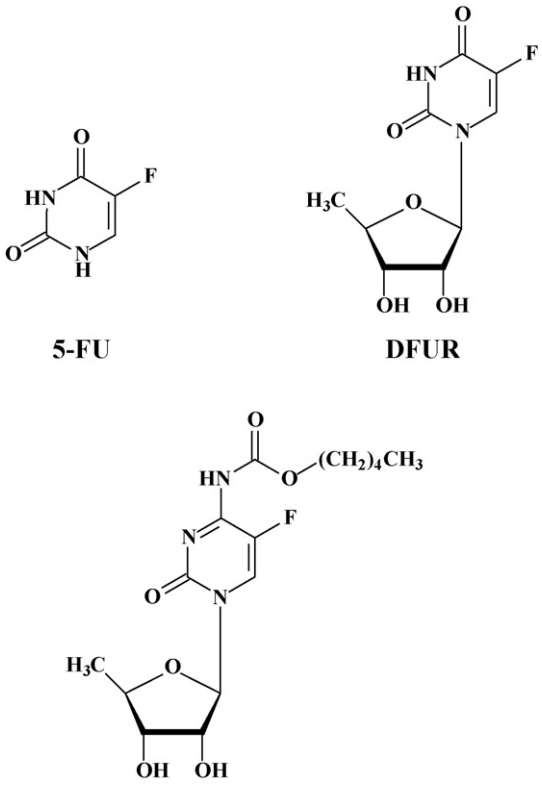


**Fig. 4 – Chemical structure of 5FU and some of its prodrugs.**

DFUR, providing direct evidence for the important role of TP in fluoropyrimidine sensitivity [5,109–111].

TP levels may also be up-regulated by cytokines and by methods used to eradicate tumors, such as chemotherapy and irradiation (see also higher) [112]. This has lead to the hypothesis that a combination of TP-inducible chemotherapy, such as cyclophosphamide and taxanes, and TP-targeted treatment, such as DFUR and capecitabine might improve the effectiveness of anti-cancer therapy [112]. A recent phase-III trial on metastatic breast cancer showed that addition of capecitabine to standard TP-inducible chemotherapy like paclitaxel, cisplatin, cyclophosphamide or irradiation results in increased response rate, time to progression and survival of patients compared to standard treatment alone [reviewed in 107]. Another phase III study comparing the efficacy of docetaxel monotherapy with docetaxel plus capecitabine showed superior time to disease progression and time to treatment failure in the combination group, suggesting that capecitabine sensitization by docetaxel might be a novel approach to breast cancer treatment [reviewed in 113]. Docetaxel and capecitabine also showed promising synergistic anti-tumor activity in phase II studies enrolling patients with advanced gastric cancer and non-small cell lung cancer [114, reviewed in 115].

Capecitabine is currently approved by the FDA for use as first-line therapy in patients with metastatic colorectal cancer when single-agent fluoropyrimidine therapy is preferred. The drug is also approved for use as a single agent in metastatic breast cancer patients who are resistant to both anthracycline- and paclitaxel-based regimens, and in combination with

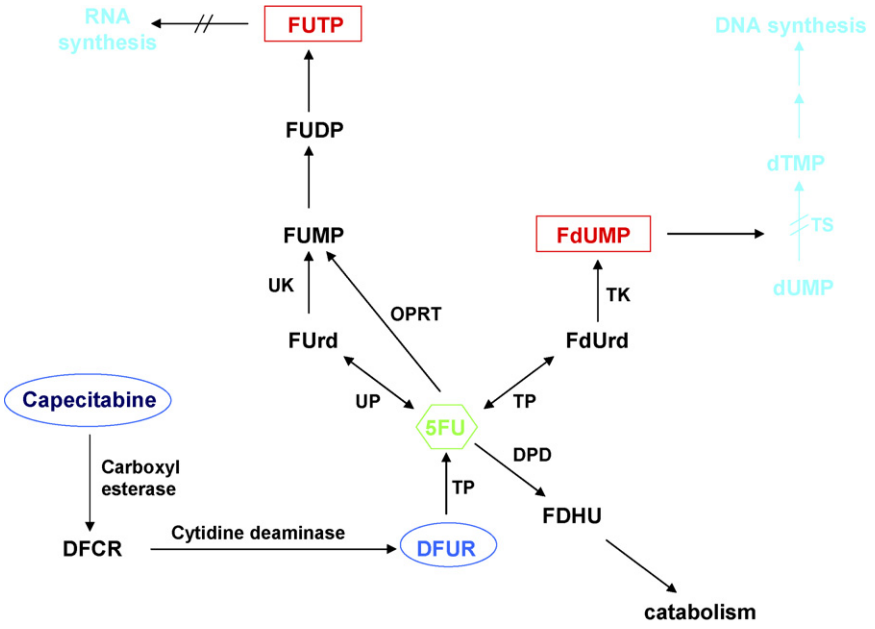


**Fig. 5 – Metabolic conversion, and mechanism of anti-tumor activity, of 5FU and its prodrugs DFUR and capecitabine. TP catalyses the conversion of DFUR to 5FU. Cytotoxicity of 5FU results from (i) incorporation of the drug into replicating RNA after its conversion to FUTP, and (ii) thymidine depletion following thymidylate synthase inhibition by FdUMP. 5FU may be directly converted to FUMP by OPRT. Alternatively, after addition of exogenous sources of 2dR1P, TP may convert 5FU to FdUrd, which is then phosphorylated to FdUMP by TK. Resistance to fluoropyrimidine therapy may result from TP deficiency or degradation of 5FU by DPD. *Enzymes*: DPD, dihydropyrimidine dehydrogenase; OPRT, orotate phosphoribosyl transferase; TP, thymidine phosphorylase; TK, thymidine kinase; TS, thymidylate synthase; UK, uridine kinase; UP, uridine phosphorylase. *Substrates:* DFCR, 5′-deoxy-5-fluorocytidine; DFUR, 5′-deoxy-5-fluorouridine; 5FU, 5-fluorouracil; FDHU, 5-fluorodihydrouracil; FdUrd, 5-fluorodeoxyuridine; FdUMP, 5-fluorodeoxyuridine monophosphate FUMP, 5-fluorouridine monophosphate; FUDP, 5-fluorouridine diphosphate; FUTP, 5-fluorouridine triphosphate; FUrd, 5-fluorouridine.**

docetaxel after failure of anthracycline-based chemotherapy [107].

## 10. Summary

In conclusion, TP is a unique enzyme that may fulfill apparently contradicting roles in cancer progression and treatment. Depending on the situation, different biological activities associated with TP might be targeted or induced. On one hand, PD-ECGF/TP contributes significantly to angiogenesis, tumor progression and metastasis, and was shown to be an independent prognostic factor in several human cancers. Yet, more studies are required to reveal the mechanism of action of TP. In particular, the signal transduction pathways, leading to TP-induced angiogenesis stimulation and protection from apoptosis, have largely remained obscure. Also, more efforts are required to identify and characterize potent TP inhibitors with anti-angiogenic and/or pro-apoptotic activities. In this sense, the outcome of the clinical studies involving TAS-102 (TFT and TPI in a 1:0.5 molar basis) will give important indications on the potential clinical use of TP inhibitors.

On the other hand, a low or deficient intratumoral TP activity has been correlated with resistance to 5FU therapy. Moreover, instead of inhibiting TP activity in tumors, most studies so far have focused on the use, and even up-regulation of TP with the purpose of tumor-selective activation of 5FU prodrugs. As a consequence, current clinical efforts are focused on the evaluation of TP-inducible therapy with TP-targeted 5FU prodrugs for cancer treatment. The oral (5FU) prodrug capecitabine, either alone or in combination with radiation, chemotherapy or specific inhibitors of growth factors or their receptors, has shown promising anti-tumor activity with manageable toxicity [116].

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