Thymidine Phosphorylase Inhibitors: Recent Developments and Potential Therapeutic Applications

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Abstract: Thymidine Phosphorylase (TPase) catalyses the reversible phosphorolysis of pyrimidine 2'-deoxynucleosides to 2-deoxyribose-1-phosphate and their respective pyrimidine bases, including the phosphorolysis of nucleoside analogues with important antiviral or anticancer properties. Moreover, TPase, identified also as the angiogenic platelet-derived endothelial cell growth factor (PD-ECGF), stimulates endothelial cell migration *in vitro* and angiogenesis *in vivo* and plays an important role in tumour progression and metastasis. Here we have summarized the most recent approaches in the search for novel TPase inhibitors together with the potential therapeutic applications of such inhibitors.

Keywords: Thymidine Phosphorylase; PD-ECGF; angiogenesis; nucleoside metabolism; anticancer drugs; Thymidine Phosphorylase Inhibitors; Inhibitor Design.

INTRODUCTION

Thymidine Phosphorylase (TPase) catalyses the reversible phosphorolysis of pyrimidine 2´-deoxynucleosides to 2-deoxy-α-D-ribose-1-phosphate and their respective pyrimidine bases, as shown in Fig. (**1**) for thymidine (**1**). TPase is involved in the salvage pathway of pyrimidine nucleotides, and its main metabolic function appears to be catabolic. TPase also catalyzes the deoxyribosyl transfer from a pyrimidine deoxynucleoside to another base to form a new nucleoside and releasing the base of the original deoxynucleoside. Besides the natural 2´-deoxynucleosides thymidine and 2´-deoxyuridine, TPase recognises several nucleoside analogues that are being used clinically as antiviral and/or anti-tumour agents, such as 5-(*E*)-(2-bromovinyl)-2´-deoxyuridine (BVDU) (**4**), 5-trifluoromethyl-2´-deoxyuridine (TFT, F_3dThd) (**5**), 5-iodo-2´-deoxyuridine (IDU) (**6**), or 5-fluoro-2´-deoxyuridine (FDU) (**7**) (Fig. **2**) [1]. Therefore, it has been proposed, already for many years, that the combination of such therapeutic agents with a TPase inhibitor could improve the biological efficacy of these nucleoside analogues.

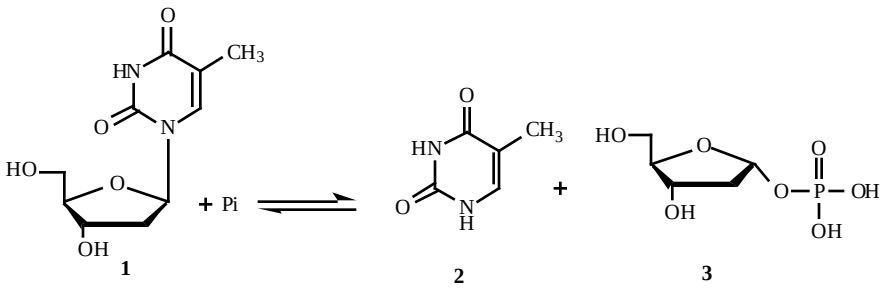

Fig. (1). Reaction catalyzed by TPase.

Interest on TPase inhibitors has been renewed in the latest years due to the implication of TPase in angiogenesis. The angiogenic protein platelet-derived endothelial cell growth factor (PD-ECGF), isolated from platelets in 1987, was shown to be identical to TPase [2]. Mutational analysis has revealed that the enzymatic activity of TPase is essential for its angiogenic effect [3,4]. *In vitro*, TPase/PD-ECGF stimulates endothelial cell migration and is therefore *in strictu senso* not an endothelial cell growth factor [5]. PD-ECGF/TPase is also known as gliostatin, initially isolated from human neurofibroma as glial growth inhibitory factor [6]. Gliostatin is neurotrophic and has a survival promoting effect on cortical neurons *in vitro* [7]. Based on chemical and biological characteristics, the three proteins (PD-ECGF, TPase and gliostatin) are considered to be identical [7]. PD-ECGF and TPase are used interchangeably throughout the literature, whereas the use of gliostatin is mainly confined to rheumatoid arthritis and neurological research [8].

TPase levels correlate well with microvessel density in breast [9], ovarian [10], colorectal [11], endometrial [12] and oesophageal cancers [13], pointing to an important role for

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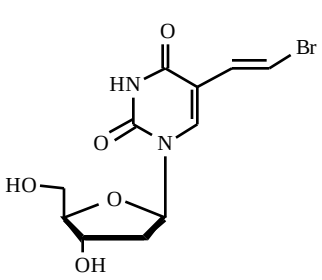
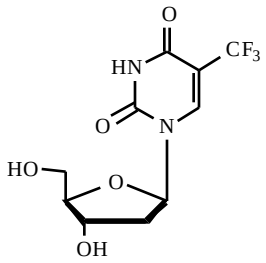
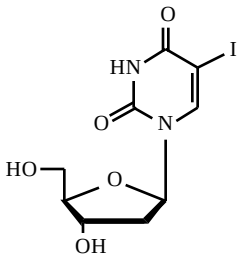


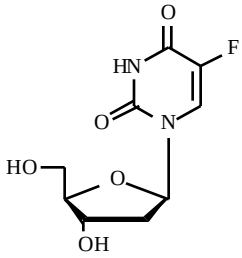
4 (BVDU)



5 (TFT, F_3dThd)



6 (IDU)



7 (FDU)

Fig. (2). Nucleoside analogues that are recognized by TPase and that are being used clinically as antiviral and/or anti-tumor agents.

this enzyme in tumour vascularisation. The mechanism by which TPase induces angiogenesis is still unclear, although significant progress has been made recently. In contrast to other angiogenesis stimulators, TPase does not contain a signal sequence required for cell secretion. Also, an endothelial cell receptor for TPase has never been identified. Therefore, it is most likely that the products of its enzymatic activity, rather than TPase itself, possess the angiogenic properties. It has been proposed that 2-deoxy-D-ribose, the dephosphorylated product derived from 2-deoxy-α-D-ribose-1-phosphate (**3**), is mostly responsible of the chemotactic activity *in vitro*, and angiogenesis *in vivo.* Several interesting reviews covering this issue have been recently published [14,15]. It has also been proposed that TPase promotes the secretion of other angiogenic factors [16].

Related or not to microvessel development, TPase is overexpressed in many solid tumours, including carcinomas of stomach [17,18], colon [19], ovary [20] and bladder [21]. TPase is also highly expressed in pancreatic cancer [22], renal carcinoma [23], breast [24] and lung cancer [25]. Moreover, TPase has been shown to inhibit tumour cell apoptosis [13]. Therefore, different observations suggest that TPase has functions unrelated to angiogenesis that affect tumour growth and metastasis [26,27].

Taking profit of the high TPase levels associated to several tumours, TPase has been involved in the activation of several prodrugs of the antitumour agent 5-Fluorouracil, such as tegafur (1-(tetrahydro-2-furanyl)-5-fluorouracil) and capecitabine (4-N-pentyloxycarbonyl-5´-deoxy-5-fluorocytidine). Under therapeutic regimens that include these prodrugs, TPase inhibitors would be incompatible. The involvement of TPase in cancer chemotherapy has been reviewed and the readers are referred to these review papers [28-31]. TPase is also overexpressed in a variety of chronic inflammatory diseases, such as human atherosclerosis [32], psoriasis [33] or rheumatoid arthritis [8].

In the present mini-review, we have tried to cover the most recent developments in the search for new TPase inhibitors, as well as the potential therapeutic applications reported for such compounds. A very brief description of the structural aspects of TPase is also included to facilitate the understanding of different approaches followed in the design of new inhibitors.

STRUCTURAL ASPECTS OF TPase

TPase is a member of the Pyrimidine Nucleoside Phosphorylase (PyNPase) family. In mammalian cells, there are, at least two enzymes belonging to this family: Thymidine Phosphorylase (TPase), and Uridine Phosphorylase (UPase). However, in lower organisms, there exists only one enzyme (PyNPase), which accepts both thymidine and uridine as the natural substrates. TPase is highly specific for 2´-deoxynucleosides of thymine and related pyrimidine bases, whereas UPase does not distinguish between ribose and deoxyribose in pyrimidine nucleosides. It is interesting to note that, in the past, the testing of substrates and/or inhibitors was quite often performed in such a manner against Pyrimidine Nucleoside Phosphorylases, making difficult to discriminate between real TPase substrates or pure inhibitors of the enzyme(s). A structure-activity relationship study of ligands of Pyrimidine Nucleoside Phosphorylases performed by Niedzwicki *et al*. [34] showed that TPase was further more exigent in the recognition of ligands that UPase. Even some previously described TPase inhibitors turned out to be UPase inhibitors. The differences between TPase and UPase are also relevant for the pharmacology of 2´-deoxynucleoside analogues with therapeutic properties. As an illustrative example, the cleavage of the glycosidic bond of 2´-deoxy-5-(trifluoromethyl)uridine (TFT, **5**) is carried out by UPase in mice and rats, while in humans, the principal cleavage enzyme is TPase [35]. Therefore, the selection of the nature

of the animal model for the evaluation of substrates and/or inhibitors of TPase is very relevant.

Structurally, TPase is a dimer made up of two identical subunits with a dimeric molecular mass ranging from 90 kDa in *Escherichia coli* to 110 kDa in mammals. There is a significant sequence homology among known members of the TPase family. For example, human TPase shares 39% of sequence identity with *E. coli* TPase. The first detailed structural information on TPase was provided by 2.8 Å resolution X-Ray crystal structure of *E. coli* enzyme [36]. Each subunit appears as a large α/β domain that contains the phosphate binding site, and, separated by a cleft, a smaller α domain where the thymidine binding site is located. The phosphate binding site is stabilized by a salt bridge with Lys-84 and several hydrogen bonds with backbone amide nitrogens and hydroxyl groups from residues lining this pocket (Ser-95, Ser-113 and Thr-123). The thymine/thymidine binding in the smaller α domain is characterized by the direct interactions of O4, N3, and O2 atoms of thymine/thymidine with Arg-171, Ser-186, and Lys-190, respectively (Fig. **3**). In this X-Ray structure, the distance between both substrates is approximately 8-10 Å, so it is generally accepted that domain closure is necessary in order to generate the active site of the enzyme. Additional determinations of the crystal structures of the *E. coli* enzyme provided evidence of domain movement to generate the functionally active TPase [37].

Other TPases structures that have been solved include *Bacillus stearothermophilus* PyNPase bound to pseudouridine and phosphate [38]. This structure supports the idea of a closed active conformation, which is the result of rigid body movement of the α and α/β domains. In early 2004 the crystal structure of Human TPase in complex with a small molecule TPase inhibitor (TPI) has been published. This structure appears to be in the active (closed) conformation [39]. Comparison with the previous solved structures, particularly with B. *stearothermophilus* PyNPase, indicates a similar overall folding. Moreover, the residues in the active site are highly conserved between the two enzymes, an observation which is important for the design and recognition of TPase inhibitors.

Although TPases of different species are highly homologous, rat TPase contains a stretch of 52 (+9) amino acids that is absent in TPase of human, mouse and *E. coli*, as shown by multiple sequence alignment (Fig. **4**). This stretch is located near the highly conserved amino acid residues that are believed to be involved in substrate binding. As a consequence, the 3-dimensional structure of

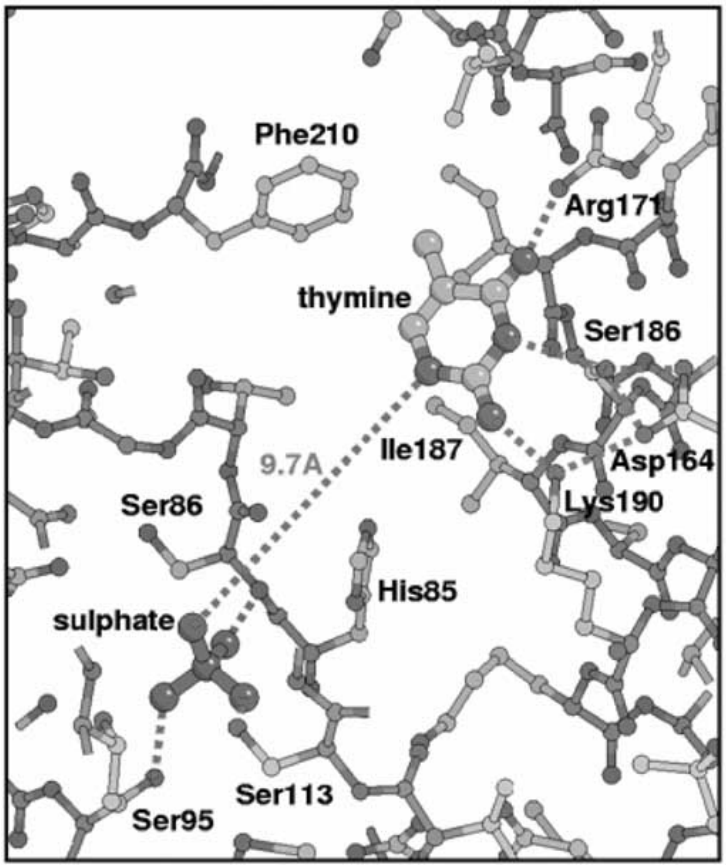


Fig. (3). Diagram showing the relationship between the thymine and the phosphate binding sites based on the crystal structure of *E. coli* TPase (Walter *et al*. [36]). Phosphate is represented as sulphate as in the X-Ray structure. Nearby residues and stabilizing interactions are shown.

the nucleoside and phosphate-binding sites in rat TPase may differ significantly from the nucleoside and phosphate-binding sites that have been described for *E. coli, B. stearothermophilus* and human TPase.

TPase INHIBITORS

First-Generation TPase Inhibitors

For more than 30 years, the reference compounds for TPase inhibition were 6-aminothymine (6AT) (**8**), 6-amino-5-chlorouracil (6A5CU) (**9**), and 6-amino-5-bromouracil (6A5BU) (**10**) (Fig. **5**), with IC_{50} values around 30 μM [40] . Pioneering work in the late sixties and early seventies had already stressed the importance to keep positions 2, 3 and 4 of the pyrimidine ring intact in order to interact with the enzyme [41]. Once the X-Ray structure of *E. coli* was solved, structural studies explained the interaction of these positions with aminoacids in the thymine/thymidine binding site, as mentioned above and shown in Fig. (**3**). Also, as a general rule, substitution at position 5 with CH_3, and particularly, Cl or Br, increases the inhibitory potency. Looking to the *E. coli* structure this can be explained by the existence of a hydrophobic pocket adjacent to C-5, lined by

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multiple sequence alignment

muis    MAAPGTPPPSA-------SGGGGG-------EPRQLPELIRLKRDGGHLREADIRNFVHAVIDGRA 52
rat     MAAPGTPPPLAPETAGADSGGGSG-------EHRQLPELIRLKRNGGHLSEADIRNFVHALMDGRA 59
human   MAALMTPGTGAPPAPGDFSGEGSQGLPDPSPEPKQLPELIRMKRDGGRLSEADIRGFVAAVVNGSA 66
E.coli  --------------------------------MFLAQEIIRKKRDGHALSDEEIRFFINGIRDNTI 34
         ::  :. . :. :..  :. .. . ...:..     *:** **:*  * : :** *: .: :.

muis    QDTQIGAMLMAIRLQGMNLEETSVLTRALAESGQQLEWP-KAWHQQLVDKHSTGGVGDKVSLVLAP 117
rat     QDTQIGAMLMAIRLQGMDLEETSVLTQALAESGQQLEWP-KAWHQQLVDKHSTGGVGDKVSLVLAP 124
human   QGAQIGAMLMAIRLRGMDLEETSVLTQALAQSGQQLEWP-EAWRQQLVDKHSTGGVGDKVSLVLAP 131
E.coli  SEGQIAALAMTIFFHDMTMPERVSLTMAMRDSGTVLDWKSLHLNGPIVDKHSTGGVGDVTSLMLGP 100
        .  **.*: *:* ::.* : *   ** *: :**  *:*     .  :*********** .**:*.*

muis    ALAACGCKV-----------------------------------------------------PMISG 144
rat     ALAACGCKVSDRLLCRSLIHSQAPPTSSLAQRQNPGLFYPPRHKFHHGIELPPSASLFHQVPMISG 190
human   ALAACGCKV-----------------------------------------------------PMISG 158
E.coli  MVAACGGYI-----------------------------------------------------PMISG 127
         :****  :                                                     *****

muis    RSLGHTGGTLDKLESIPGFGVTQSPEQ---------MLHILEEVGCCIVGQSAKLVPADGILYAAR 188
rat     RSLGHTGGTLDKLESIPGFSVTQSPEQKSMKVSALQMLQILEEVGCCIVGQSEKLVPADGILYAAR 256
human   RGLGHTGGTLDKLESIPGFNVIQSPEQ---------MQVLLDQAGCCIVGQSEQLVPADGILYAAR 202
E.coli  RGLGHTGGTLDKLESIPGFDIFPPDDNR---------FREIIKDVGVAIIGQTSSLAPADKRFYATR 171
        *.*****************.:  . ::          :  ::.:.* .*:**: .*.***  :**:*

muis    DVTATVDSVPLITASILSKKAVEGLSTLVVDVKFGGAAVFPDQEKARELAKMLVRVGVSLGLKVAA 254
rat     DVTATVDSVPLITASILSKKAVEGLSTLVVDVKFGGAAVFPDQEKARELAKMLVRVGMGLGLQVAA 322
human   DVTATVDSLPLITASILSKKLVEGLSALVVDVKFGGAAVFPNQEQARELAKTLVGVGASLGLRVAA 268
E.coli  DITATVDSIPLITASILAKKLAEGLDALVMDVKVGSGAFMPTYELSEALAEAIVGVANGAGVRTTA 237
        *:******:********:** .***.:**:***.*..*.:*  * :. **: :* *. . *::.:*

muis    ALTAMDNPLGRSVGHTLEVEEALLCLDGAGP-PDLRDLVIRLGGAILWISGQAETQDQGAARVAAA 320
rat     ALTAMDNPLGRNVGHTLEVEEALLCLDGAGP-PDLRDLVIRLGGAILWLSGQAETQDQGAARVAAA 388
human   ALTAMDKPLGRCVGHALEVEEALLCMDGAGP-PDLRDLVTTLGGALLWLSGHAGTQAQGAARVAAA 334
E.coli  LLTDMNQVLASSAGNAVEVREAVQFLTGEYRNPRLFDVTMALCVEMLISGKLAKDDAEARAKLQAV 303
         ** *:: *.  .*:::**.**:  : *    * * *:.  *   :*  .  *  : :. *:: *.

muis    LDDGSARRRFQLMLSAQGVDPGLAKALCSGSPTQRRQLLPHAREQEELLAPADGIVECVRALPLAR 385
rat     LDDGSALHRFQLMLSAQGVDPGLARALCSGSPTQRRQLLPHARKQEELLSPADGIVECVRALPLAC 453
human   LDDGSALGRFERMLAAQGVDPGLARALCSGSPAERRQLLPRAREQEELLAPADGTVELVRALPLAL 399
E.coli  LDNGKAAEVFGRMVAAQ-KGP-------TDFVENYAKYLPTAMLTKAVYADTEGFVSEMDTRALGM 365
        **:*.*   *  *::**. .*. : : .:.   :  : ** *   : : : ::* *. : : .*.

muis    VLHDLGAGRSRAGQPIRPGVGAEVLVDVGQCLSRGTPWLRVHLDGPALSSQQRRTLQGALVLSDRA 451
rat     VLHELGAGRSRAGQPIRPGVGAELLVDVGQWLSRGTPWLRVHLDGPALSSQQRRTLLGALVLSDRA 519
human   VLHELGAGRSRAGEPLRLGVGAELLVDVGQRLRRGTPWLRVHRDGPALSGPQSRALQEALVLSDRA 465
E.coli  AVVAMGGGRRQASDTIDYSVGFTDMARLGDQVDGQRPLAVIHAKDENNWQEAAKAVKAAIKLADKA 427
        .:  :*.** :*.:.:  .**   :. :*: :   *   :* ..        :::  *: *:*:*

muis    PFKVPSPFAELVLPPTIAQP 471
rat     PFKAPSPFAELVLPPTTP-- 537
human   PFAAPLPFAELVLPPQQ--- 482
E.coli  PESTPTVYRRISE------- 440
        *  .*  : .:
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Fig. (4). Multiple sequence alignment of mouse, rat, human and *E. coli*. TPase, according to EMBL-EBI database: * the residues in that column are identical in all sequences in the alignment; . conserved substitutions have been observed; : semi-conserved substitutions are observed.

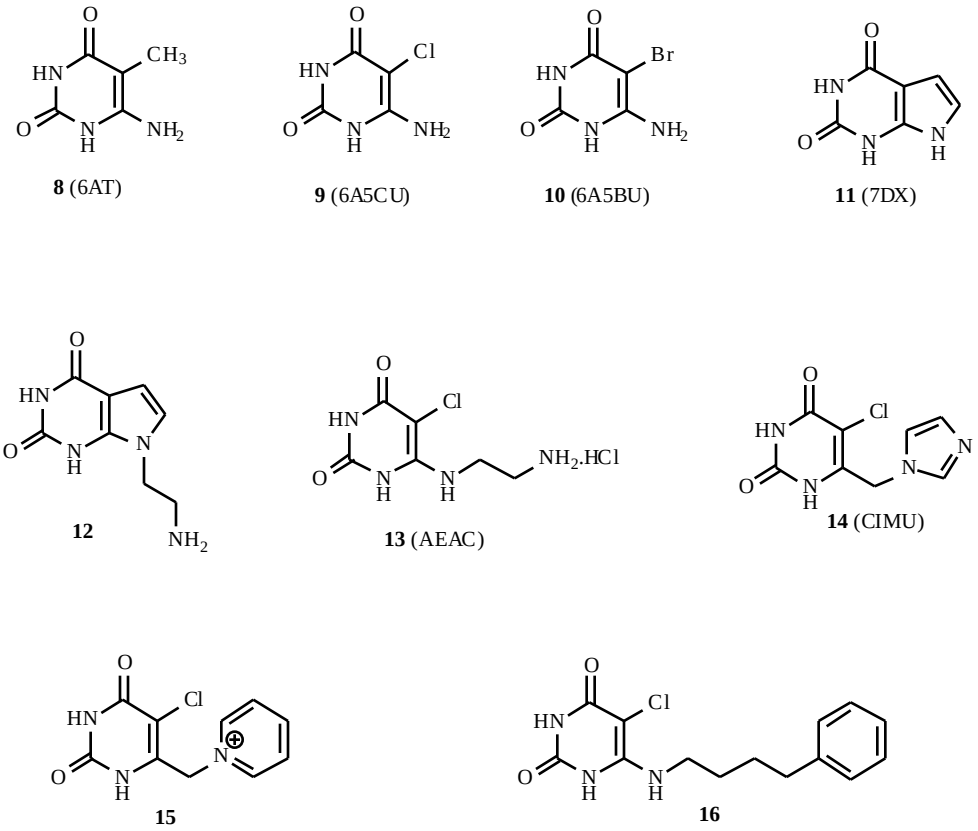


Fig. (5). First generation TPase inhibitors and inhibitors based on 6-aminouracil.

Phe210, that could easily fit these small substituents, while the non substituted compounds lack this additional lipophylic interaction. Also, based on the previously synthesized compounds, there seems to be a hydrophobic cavity around position 6 of thymine as well. So, most efforts have been directed to fulfil this cavity. These facts explain why structurally most TPase inhibitors are 6-amino or 6-methyleneamino uracil derivatives.

TPase Inhibitors Based on 6-Aminouracil

Based on the coordinates of the *E. coli* enzyme, the well-known TPase inhibitors 6-aminothymine (**8**) and 6-amino-5-bromouracil (**10**) were modelled in the active site at the position of thymine. Novel potential inhibitors were designed, including the addition of a second ring on the pyrimidine base, intended to create extra stabilising interactions by filling the apparent space near the ring of residue Phe210 (a Val residue in the human TPase structure). This resulted in 7-deazaxanthine (7DX) (**11**) (Fig. **5**), a purine derivative showing an IC_{50} value similar to the reference compounds against the *E. coli* enzyme [42]. Other 7-deazaxanthine derivatives like the 7-(2-aminoethyl)-deazaxanthine (**12**) synthesized by Hirota *et al*. [43] showed a similar IC_{50} value (44 μM) against human placenta TPase.

The main purposes in the synthesis of new TPase inhibitors derived from 6-aminouracil or analogues described in the latest years have been to improve the inhibitory potency against TPase, but also to increase their solubility compared to the parent 6A5BU. Efforts in this sense include 6-(2-aminoethylamino)-5-chlorouracil (AEAC) (**13**) (Fig. **5**), that was found to be 65-fold more potent against human TPase than 6A5BU [44]. Comparison with the corresponding 6-aminoethanol derivative that was inactive, stressed the importance of the basicity of the substituent at position 6 in the inhibitory potency. Other substitutions included in this paper gave rise to 5-chloro-6-(1-imidazolyl-methyl)uracil (CIMU) (**14**) that was similarly active to AEAC against TPase, but resulted also in a marginal inhibition of UPase [44].

Water soluble pyridinium-substituted uracil derivatives have also been synthesized and tested [45]. Introduction of the pyridinium ring directly on the 6-position of the pyrimidine base resulted in inactive compounds, while the corresponding 6-methylenepyridinium compounds, like (**15**) (Fig. **5**), were similarly active to 6A5BU. Also a series of 6-(phenylalkylamino)uracil derivatives have been prepared, the most potent being 6-(4-phenylbutylamino)uracil (**16**) (Fig. **5**) with an inhibitory potency slightly higher than 6-aminothymine [46]. Interestingly, in this latest series of compounds, the corresponding 5-chloro- or 5-methyl analogues were less active than the unsubstituted compounds, what constitutes an exception to the general behaviour of TPase inhibitors.

TPI and Analogues

The most extensive efforts in exploring substitutions at position 6 of the above mentioned compounds have been performed by Taiho Pharmaceutical Co., Ltd. A preliminary report on several 6-substituted-5-chlorouracil derivatives were reported in 2000 [47], including the very potent TPase

inhibitor 5-chloro-6-[1-(2-iminopyrrolidinyl)methyl]uracil hydrochloride (TPI) (**17**) (Fig. **6**). Only recently full details on the discovery process of the lead compounds of TPI have been disclosed [48,49].

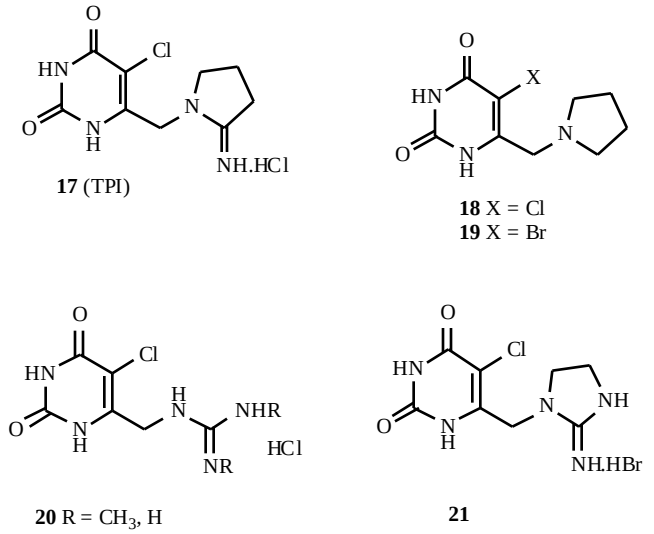


Fig. (6). TPI and analogues.

The development of these TPase inhibitors has been predominantly driven by the potential of TPase inhibitors to improve the potency of antitumour 2´-deoxynucleosides, and, in particular, the antitumour effects of 2´-deoxy-5-(trifluoromethyl)uridine (F_3dThd) (**5**). The final candidate drugs should be selected not only based on their *in vitro* inhibitory activity against TPase, but also by a reasonable pharmacokinetic profile as well as by their ability to increase the plasma concentrations of the antitumour drug, particularly F_3dThd, in mice. Several series of 6-methyleneamino uracil derivatives were synthesised and evaluated against human TPase, showing that the hydrophobic region contacting position 6 of thymine in human TPase does not allow very bulky substituents. 6-Substituted uracil derivatives like AEAC (**13**) and CIMU (**14**) were also included in the SAR studies against human TPase, although AEAC (**13**) gave lower inhibition than previously reported. The best results were obtained with 6-methylenepyrrolidine uracil derivatives substituted at position 5 of the pyrimidine ring with Cl or Br (compounds **18** and **19**), (IC_{50} values of 2.2 and 0.51 μM, respectively) (Fig. **6**). [49]. Thus, compound **19** was selected for *in vivo* evaluation. However, disappointing toxic effects in mice stimulated the search for novel series of 6-methylene bridged uracil derivatives possessing a guanidine or amidino function [48]. Interestingly, compound **20** with a *N*-methylguanidine substituent showed even more potent activity that the initial lead compound with a pyrrolidine moiety (**18**). Further work in this series lead to compounds **21** and **17** (TPI) with IC_{50} values as low as 13 and 35 nM, respectively, against human TPase. Moreover, both compounds showed a good profile in the preliminary pharmacokinetics experiments. However, synthetic reasons discarded compound **21** for further development, and TPI was selected for clinical trials [48]. It is interesting to note than TPI is a highly specific TPase inhibitor and does not inhibit human and mouse UPase.

Design of TPase Inhibitors Based on the Structural Information on TPase

Till early 2004, the structure of human TPase had not been solved. Previous structural determinations include two bacterial analogues: *E. coli* and *B. stearothermophilus*, that share approximately 40% sequence identity with human TPase. The coordinates of PyNPase of *B. stearothermophilus* co-crystalised with pseudouridine in the closed conformation were used to build a homology model of human TPase. Based on the potent inhibitory activity of TPI (**17**), *N*-(2,4-dioxo-1,2,3,4-tetrahydro-thieno[3,2-*d*]pyrimidin-7-yl)guanidines, like **22** (Fig. **7**), were designed [50]. Flexible docking experiments of the designed compounds in the constructed model of human TPase showed good agreement with TPI binding. However, once the compounds were synthesized and tested, the experimental binding affinities were around 60 μM, which

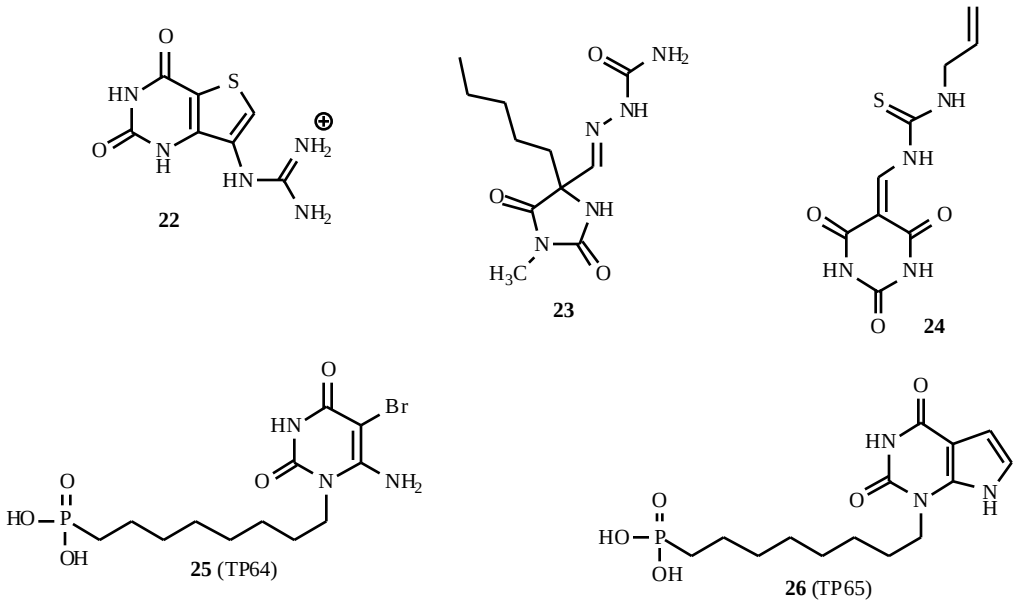


Fig. (7). TPase inhibitors designed based on structural information on TPase and multisubstrate inhibitors.

are far from the 30 nM of TPI. A desolvation penalty for the hydrophilic guanidine moiety has been proposed to contribute to the reduced binding affinity [50].

The *E. coli* structure in the open-cleft conformation has also been used to construct a homology model of human TPase [51]. The active site in this model was used for a structure-based approach to screen for alternative non-nucleobase derived inhibitors and to identify new scaffolds active against human TPase [52]. After performing computational screening of the National Cancer Institute database of anticancer compounds, thirteen compounds were finally selected to be included in the TPase assay. From these compounds, **23** and **24** afforded the best inhibition against *E. coli* TPase, with IC_{50} values of 20 and 95 μM, respectively (Fig. **7**). Analysis of the binding mode of compound **23** indicated that the methylhydantoin moiety was buried deep in the active-site cleft, close to the phosphate binding site. On the other hand, compound **24** was predicted to interact at the thymine-binding site. Although the starting level of inhibition is still weak, ligand **23** represents a novel scaffold unrelated to the uracil moiety that could be useful for the design of new inhibitors.

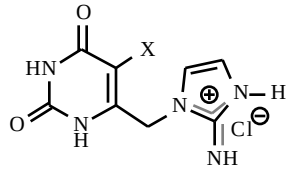
Multisubstrate Inhibitors

The structure of *E. coli* TPase in the open conformation has also been used for the design of multisubstrate inhibitors. In this open conformation, the distance between the thymine/thymidine binding site and the phosphate binding site is approximately 8-10 Å (Fig. **3**). Based on these structural features, several series of compounds containing a thymine base, interacting at the nucleoside binding site, a spacer of 6 to 9 atoms, and a phosphonate moiety, to interact at the phosphate binding site, were synthesized and tested [53]. Further replacement of the thymine ring in the most active compounds by 6-A5BU or 7-deazaxanthine afforded compounds TP64 (**25**) and TP65 (**26**) (Fig. **7**), respectively, with IC_{50} values against *E. coli* or human TPase ranging between 20 and 80 μM [53]. Detailed kinetics experiments revealed that these compounds inhibited TPase in a purely competitive or mixed fashion when thymidine but also when inorganic phosphate were used as variable substrates, suggesting that TP64 and TP65 are able to interact both at the thymine/thymidine and the phosphate binding sites, as originally designed [54]. These compounds, being water-soluble due to the presence of the phosphonate moiety, represent the first described examples of multisubstrate inhibitors of TPase.

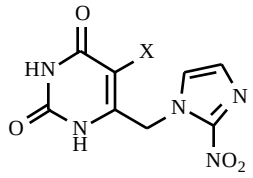
Prodrugs of TPase Inhibitors

As TPase is highly expressed in different tumours, it has been proposed that there should be an advantage in generating the TPase inhibitors only or predominantly at the tumour site. The tumour environment is characterized by hypoxia that may cause the bioreductive activation of nitro groups to amino derivatives. With this idea, nitroimidazolylmethyluracil prodrug derivatives have been designed. Both the nitro and the corresponding amino derivatives, resulting from the bioreductive activation, were modelled in the active site structure of *E. coli* TPase or the human TPase, obtained through a similarity model [55]. It was predicted that the enzyme could discriminate between the nitro and the amino forms. To test this hypothesis, both series of compounds were synthesized and tested for their inhibitory potential against *E. coli* TPase. The results obtained indicated that 5-halo-2-amino-imidazolyl-methyluracils (compounds **27** and **28**) (Fig. **8**) were very potent inhibitors of *E. coli* TPase (IC_{50} close to 20 nM), while the corresponding 2′-nitroimidazolylmethyluracil prodrugs (compounds **29** and **30**) were 1000-fold less active (IC_{50} around 20 μM) [55].

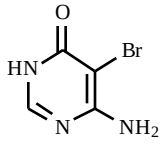
Another activation approach of prodrugs based on well-established TPase inhibitors makes use of the high levels of xanthine oxidase (XO) in hypoxic tissues, like breast, colorectal or brain tumours. Moreover, xanthine oxidase is endowed with a broad substrate specificity. A series of potential tumour-selective XO-activated prodrugs of known TPase inhibitors such as compounds **31**, **32** and **33** (Fig. **8**), were designed, synthesized and tested [56]. The results



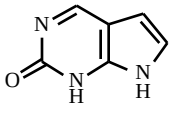
27 X = Cl
28 X = Br



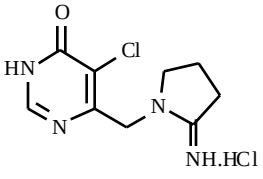
29 X = Cl
30 X = Br



31



32



33

Fig. (8). Prodrugs of TPase inhibitors.

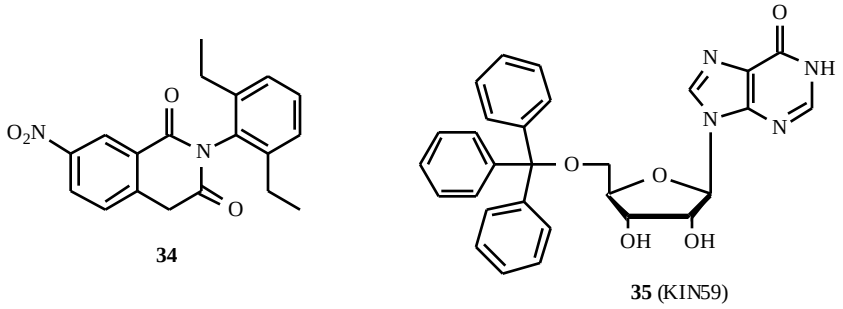


Fig. (9). TPase inhibitors interacting at a non-substrate binding site.

obtained suggest that these prodrugs are oxidised by xanthine oxidase at C-2 and/or C-4 of the uracil ring to generate the corresponding TPase inhibitors 6A5BU, 7DX and TPI, respectively. Also, as expected, the prodrugs (**31**, **32** and **33**) as such are poor TPase inhibitors. It should be noted that these prodrugs lack the carbonyl function at positions C-2 or C-4 of the pyrimidine ring, stressing the importance of these positions for the interaction of the compounds at the TPase active site, as already mentioned. The two prodrug approaches represent new opportunities to develop tumour-targeted inhibitors of TPase.

TPase Inhibitors Interacting with a Non-Substrate Binding Site

Almost all the above mentioned compounds interact with TPase at the substrate(s) binding site(s), in particular at the thymine/thymidine binding site. However, there are now examples of compounds that seem to explore new binding sites on TPase. It has been described that *N*-phenyl homophtalimide derivatives like **34** (Fig. **9**) with an IC_{50} value of 246 μM, showed mixed-type competitive inhibition, suggesting the existence of at least two binding sites for this compound on TPase, one of them being the substrate binding site [57].

We have recently reported on the inhibitory activity of the purine riboside derivative KIN59 (5´-*O*-tritylinosine) (**35**) (Fig. **9**) against human and *E. coli* recombinant TPase, with IC_{50} values of 67 and 44 μM [58]. In contrast to previously described TPase inhibitors, KIN59 compete neither with the pyrimidine nucleoside nor with the phosphate-binding site of the enzyme, but non-competitively inhibits TPase when thymidine or phosphate is used as the variable substrate. These findings point to the existence of a novel, till now unknown, allosteric binding site at TPase, different from the thymidine and phosphate-binding sites. It cannot be excluded that such allosteric site also plays a role in the biological properties of TPase, as will be discussed below.

THERAPEUTIC POTENTIAL OF TPase INHIBITORS

As mentioned in the Introduction, TPase inhibitors could be useful to prevent the degradation of nucleoside analogues with anticancer and/or antiviral properties. However, the implication of TPase in angiogenesis has opened additional possibilities for TPase inhibitors. In this section, we will try to summarize what has been published recently concerning the potential therapeutic applications of TPase inhibitors.

The well-established TPase inhibitor 6-amino-5-chlorouracil (6A5CU, **9**) was used to validate the role of TPase in angiogenesis. 6A5CU was administered to mice orally for 10 days from 1 day after implantation of sponges soaked with TPase. At 300 mg/kg/day, 6A5CU almost completely inhibited the angiogenic effect of TPase, but did not significantly inhibit the angiogenic response induced by other factors (i.e bFGF) [3].

Compound TPI (**17**), being 1000-fold more inhibitory against TPase than 6A5CU, has also been used to establish the role of TPase in tumour growth and angiogenesis. The effect of TPI on angiogenesis in human epidermoid carcinoma KB cells transfected with PD-ECGF/TPase cDNA (KB/TP) has been investigated using the mouse dorsal air sac assay model [59]. In this model, at doses of 50mg/kg/day, TPI completely suppressed angiogenesis induced by the KB/TP tumour cells. The effect of TPI on the growth rate of KB/TP tumour cells xenografted into nude mice was also investigated. At a dose of 50 mg/kg/day, TPI considerably decreased the growth rate (30.8% compared to control) and significantly increased the apoptotic index in KB/TP tumours. Further studies afforded evidence that TPI suppressed chemotactic motility and basement membrane invasion of KB/TP cells. In nude mice, oral administration of TPI suppressed liver metastases by highly metastatic KB/TP cells [60]. In a different study, serous adenocarcinoma cells, which have high levels of TPase expression, were used to induce angiogenesis in a dorsal air sac model in mice. Oral administration of TPI significantly inhibited angiogenesis in this model, suggesting that the angiogenic potency of ovarian cancer cells is controlled by TP/PD-ECGF [10]. All together, these data indicate that TPI inhibits TPase-induced angiogenesis, increases the proportion of apoptotic cancer cells in TPase-positive tumours, suppresses the growth of these tumours and might be considered as a novel potential antimetastatic agent.

The effect of other TPase inhibitors in different experimental models of angiogenesis has also been evaluated. One of these models is the chick chorioallantoic membrane (CAM) assay. In this assay, TPase caused a dose-dependent stimulation of angiogenesis, which was significantly inhibited by the addition of 250 nmol TP65 (**26**), that is at a dose proved to be non-toxic for the developing chick embryo [61]. *In vitro*, TP65 completely inhibited TPase-induced formation of microvascular sprouts from endothelial cell aggregates in a three-dimensional fibrin

gel. Very recently, we have reported the effect of the allosteric TPase inhibitor KIN59 (**35**) in the CAM assay [58]. Although the IC_{50} value of KIN59 in the enzymatic assay against TPase was quite similar to that of the previously described multisubstrate inhibitor TP65, KIN59 was far more active in the CAM assay. The anti-angiogenic effect of KIN59 was not accompanied by inflammation or any visible toxicity. The data suggest that the angiogenic activity of TPase may not solely be directed through its functional thymidine and phosphate binding sites, but that other regulatory (allosteric) site(s) may be playing an important role in the mechanism of TPase-triggered angiogenesis stimulation.

TPase-transfected carcinoma cells and human monocyte cells, which constitutively express high levels of TPase, induced human umbilical vein endothelial cell (HUVEC) migration in a co-culture assay. CIMU (**14**) inhibited tumour-cell and monocyte-induced migration. By contrast, a neutralizing antibody to TPase had no effect on cell-stimulated HUVEC migration, although it completely inhibited migration induced by purified TPase [62], which indicates that the intracellular metabolism of thymidine is sufficient to stimulate HUVEC chemotaxis. The above-mentioned observations illustrate the role of TPase inhibitors as a tool to better understand the mechanism(s) by which TPase is involved in angiogenesis and tumour growth.

As mentioned several times along this article, TPase inhibitors could be helpful to prevent the degradation of nucleoside analogues with anticancer and/or antiviral properties. The development of TPI and its analogues were particularly performed to enhance the plasma concentration of TFT (**5**) in monkeys and to potentiate the *in vivo* antitumour activity of TFT after combined oral administration [48,49]. With these premises, a combination of TFT and TPI in a 1:0.5 mixture on a molar basis, designated as TAS-102, has entered phase I clinical trials for colorectal and breast cancer patients. It has been proposed that the expected antitumour activity of this drug combination can be ascribed to inhibition of thymidylate synthase by the corresponding TFT monophosphate and/or incorporation of the TFT-triphosphate into DNA, leading to tumouricidal effects [63]. However it is important to remember that the presence of TPI in this drug combination may not only increase the levels of the antitumoural nucleoside TFT, but that TPI itself may have an antitumour effect based on its anti-angiogenic activity and its potential to inhibit liver metastasis [64].

PERSPECTIVES

Interest on TPase inhibitors has been considerably renewed in the latest years and different strategies have been followed to design new and potent inhibitors. However, in our opinion, there is still room for further improvement, and in the following years the development of new compounds, particularly exploring leads different from the traditional 6-substituted uracil compounds, will emerge. It is very important and encouraging that one of the 6-substituted uracil compounds, TPI, has now entered clinical trials in the combination with TFT, designated as TAS-102. The outcome of these studies will be extremely helpful to establish the clinical relevance of a potent TPase inhibitor.

New perspectives have also been recently opened for the development of new compounds. Very recently, the structure of human TPase co-crystalised with TPI has become available [39]. The structure appears to be in the active (closed) conformation. It is proposed that TPI binds as a zwitterion, mimicking the transition state during the phosphorolysis reaction catalyzed by TPase, and this could help to explain its high potency and selectivity [39]. Moreover, in the crystal structure, phosphate is absent, indicating that phosphate is not necessary for enzyme inhibition and generation of the closed conformation. According to the structural coordinates, it is proposed that the highly conserved His116 acts as the proton donor to the N-1 amido position of thymidine for phosphorolysis [39]. Interestingly, the role of this same amino acid (His-85 in *E. coli*) in the catalytic mechanism has also been recently studied by targeted molecular dynamics and quantum mechanical calculations [65]. The results obtained revealed that this His residue could be involved in the protonation of the pyrimidine base at the O-2 position, instead of N-1, yielding the enol tautomer of the base that spontaneously tautomerises back to the more energetically stable keto form [65]. The transition state of TPase has also been recently determined using kinetic isotope effect analysis, followed by computer modelling [66]. According to the results obtained, it is hypothesized that the strong affinity of TPase for TPI could be explained by considering that the iminopyrrolidine ring is not acting as an oxacarbenium ion, but as a phosphate chelator so that the ion-pair complex resembles the transition state proposed based on kinetic isotope effect analysis [66]. These different views on the catalytic mechanism of TPase proves that still much can be learned on the molecular mechanism of TPase enzymatic activity and that more insights herein will allow rational design of novel potent and selective TPase inhibitors. All these studies on the catalytic mechanism of TPase as well as the unexplored areas that have been described in the co-crystallization of TPI with TPase [39], open the possibilities for the design of novel TPase inhibitors. Although most inhibitors of TPase seem to compete with the substrate-binding site of the enzyme, the recent discovery of a selective non-competitive inhibitor of TPase targeted at a new (hitherto unknown) allosteric binding site [58] may open interesting perspectives for the development of entirely novel classes of TPase inhibitors with structures entirely unrelated to the natural substrates of the enzyme. Such compounds may also contribute to reveal and unravel the molecular role of TPase in angiogenesis and other biological processes such as apoptosis and tumour cell metastasis.

The interest on TPase based on its clinical implications, the first results on the clinical trials where a TPase inhibitor is being tested, and our better knowledge of the structural requirements of this enzyme, will probably push the development of new TPase inhibitors as an active area of research for medicinal chemists in the next years.

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