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Fluorescence and computational studies of thymidine phosphorylase affinity toward lipidated 5-FU derivatives

R. Lettieri [a], M. D'Abramo [b], L. Stella [a], A. La Bella [b], F. Leonelli [c], L. Giansanti [d,e], M. Venanzi [a], E. Gatto [a,*]

[a] Dipartimento di Scienze e Tecnologie Chimiche, Università degli Studi di Roma Tor Vergata, Roma, Italy
[b] Dipartimento di Chimica, Università degli Studi di Roma La Sapienza, Roma, Italy
[c] Dipartimento di Biologia Ambientale, Università degli Studi di Roma "La Sapienza", Roma, Italy
[d] Dipartimento di Scienze Fisiche e Chimiche, Università degli Studi dell'Aquila, Aquila, Italy
[e] CNR, Istituto di Metodologie Chimiche, Monterotondo Scalo, Rome, Italy

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ABSTRACT

Thymidine phosphorylase (TP) is an enzyme that is up-regulated in a wide variety of solid tumors, including breast and colorectal cancers. It is involved in tumor growth and metastasis, for this reason it is one of the key enzyme to be inhibited, in an attempt to prevent tumor proliferation. However, it also plays an active role in cancer treatment, through its contribution in the conversion of the anti-cancer drug 5-fluorouracil (5-FU) to an irreversible inhibitor of thymidylate synthase (TS), responsible of the inhibition of the DNA synthesis. In this work, the intrinsic TP fluorescence has been investigated for the first time and exploited to study TP binding affinity for the unsubstituted 5-FU and for two 5-FU derivatives, designed to expose this molecule on liposomal membranes. These molecules were obtained by functionalizing the nitrogen atom with a chain consisting of six (**1**) or seven (**2**) units of glycol, linked to an alkyl moiety of 12 carbon atoms. Derivatives (**1**) and (**2**) exhibited an affinity for TP in the micromolar range, 10 times higher than the parent compound, irrespective of the length of the polyoxyethylenic spacer. This high affinity was maintained also when the compounds were anchored in liposomal membranes. Experimental results were supported by molecular dynamics simulations and docking calculations, supporting a feasible application of the designed supramolecular lipid structure in selective targeting of TP, to be potentially used as a drug delivery system or sensor device.

1. Introduction

Thymidine phosphorylase (TP) is a fundamental enzyme in the metabolism of pyrimidine [1–3]. It belongs to the family of pentosyltransferases and catalyzes the reversible phosphorolysis of thymidine and other pyrimidine 2′-deoxyribosides [3]. TP represents the conjunction between pyrimidine anabolism and catabolism as it regulates the amount of thymine for DNA synthesis, by increasing its production or, if it is in excess, by sending it to degradation through nitrogenous bases catabolism. This enzyme promotes angiogenesis, a process that leads to the formation of new capillaries from pre-existing blood vessels [4,5]. It has been demonstrated that TP up-regulation is closely related to the onset of a wide range of cancers (breast, ovarian, colorectal and esophageal tissues) [5].

TP is a homodimer consisting of two identical subunits [3]. X-ray studies on *E. coli* TP at 2.8 Å resolution [6], revealed that each monomer is composed of a small α-domain, made of 7 α-helices (Fig. 1, pink), and a larger mixed α/β domain, composed of a central β-sheet domain surrounded by α-helices (Fig. 1, pale blue), with a total of 440 amino acids. The two domains are separated by a large cleft. The phosphate binding site is located across a cleft in the α/β domain, while the thymidine binding site is located in the small α-domain (Fig. 1, red). The 2′-deoxyribose moiety instead is located in a small tunnel that connects the two binding pockets. Recent crystallographic data showed that a significant relative mobility of the protein domains takes place, and this is required for catalysis [3,6,7].

TP, being a key enzyme in the metabolism of pyrimidine, is a relevant target in the development of new cancer therapeutic strategies [8]. However TP is also responsible of the activation patterns of 5-FU, which is a pyrimidine analogue widely used as anticancer drug [9–13]. 5-FU becomes cytotoxic after the conversion, catalyzed by TP, to 5-fluoro-2′-deoxyuridine (FdUrd), which is further converted by thymidine kinase to 5-fluoro-2′-deoxyuridine 5′-monophosphate (FdUMP). FdUMP is an irreversible inhibitor of thymidylate synthase (TS), thus preventing the synthesis of DNA [14]. 5-FU is administered orally as a prodrug (capecitabine), due to the gastrointestinal toxicity of FdUrd, in order to generate 5-FU, and consequently the FdUrd, at the tumor

* Corresponding author.
E-mail address: emanuela.gatto@uniroma2.it (E. Gatto).

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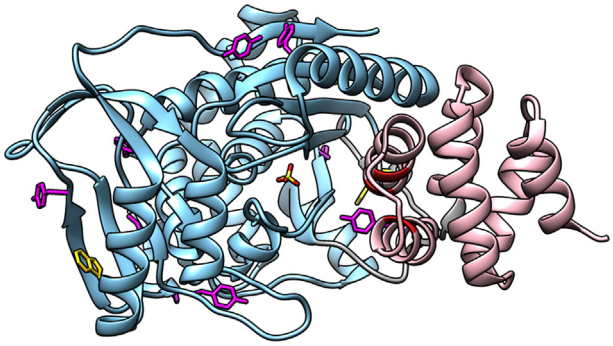
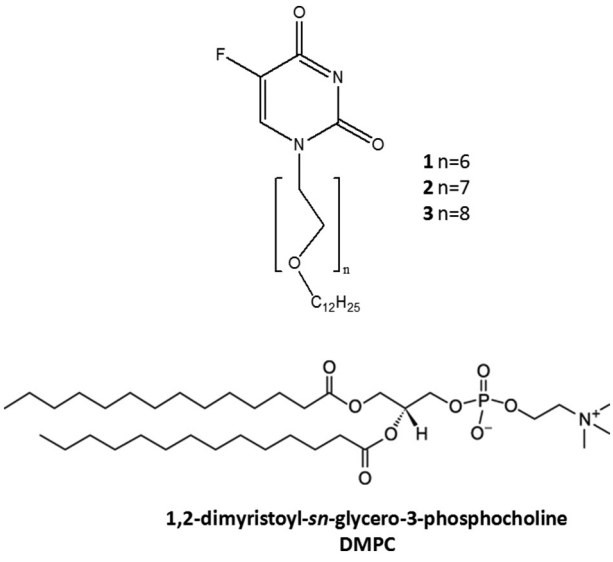


Fig. 1. Ribbon representation of the *E. coli* TP protein structure taken from the Protein Data Bank (PDB ID: 4EAF). The intrinsically fluorescent residues (two Trp and nine Tyr) are shown in stick representation. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

site [5]. Responsible for this activation is again the TP enzyme, which has been defined, for this reason, an enemy and friend of cancer disease [5,9].

Recently, some of us have designed and characterized three 5-FU derivatives that spontaneously insert into phospholipid liposome bilayers (amphiphiles **1**–**3**, Scheme 1) [15]. In these derivatives, the N^3 position of 5-FU was alkylated with a chain consisting of six (**1**), seven (**2**) or eight (**3**) units of ethylene glycol linked to an alkyl chain of 12 carbon atoms [15]. The hydrophobic $CH_3(CH_2)_{11}$ chain was designed to ensure the anchorage of the 5-FU derivatives to the phospholipid bilayer of a liposome. Glycol units have been inserted in order to act as spacers to avoid inhibition of 5-FU/enzyme interactions by the steric hindrance of the liposomes. Kinetic experiments and docking simulations performed between TP and the derivative **3** showed that this derivative had an enhanced capability of binding TP compared to 5-FU, probably due to the presence of an additional hydrogen bond between the arginine 357 residue and the glycol chain [16]. However, experimental data concerning the shorter derivatives are missing. Furthermore, 5-FU derivatives have a great potential once anchored in liposomes, as drug nano-carriers, or as sensors [17,18], due to the 5-FU specificity for the proteins involved in DNA synthesis (TP, dihydropyrimidine dehydrogenase (DPD) and TS) and due to the high concentration of these molecules at tumor sites. To this aim, the determination of the derivative's affinity toward the target proteins is essential.



Scheme 1. Chemical structure of the 5-FU derivatives and liposome component.

Fluorescence spectroscopy is a widely used technique in protein binding studies [19,20], as the binding of a ligand causes in general a change in the protein three-dimensional structure, which has an effect on the environment of the protein fluorophores, producing a measurable change in the fluorescence spectrum, such as a shift in the wavelength of maximum emission or changes in emission intensity [20–23]. Interestingly, each *E. coli* TP subunit is characterized by the presence of several fluorescent amino acids: two tryptophan (Trp 72, 410) and nine tyrosine (Tyr 108, 168, 214, 267, 330, 333, 344, 379, 435) residues (Fig. 1) [6]. Intrinsic protein fluorescence, using tryptophan (Trp) as a reporter, provides a sensitive measure of protein tertiary structure [21,22]. However, despite the importance of TP protein in tumor proliferation and diagnosis, and the consequent need for TP inhibitors and drugs, in the literature there is no fluorescence characterization of TP protein nor ligand binding using this versatile technique.

In the present work, TP fluorescence has been characterized by steady-state and time-resolved measurements. With the same techniques we have quantified TP binding affinity for the unsubstituted 5-FU molecule, which has never been determined before. MD simulations have been performed to study 5-FU binding, and theoretical calculations have been compared to the experimental results. By steady-state and time-resolved measurements we have also determined the TP binding constant values of the shorter derivatives **1** and **2**, alone or when embedded into 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) liposomes (Scheme 1).

2. Experimental and Theoretical Methods

2.1. Materials

The commercially available TP from *E. coli* was employed because of its similarity to the human enzyme. The molecular mass of the dimer is 90 kDa for the *E. coli* TP [24] and 110 kDa for the human TP [25]. *E. coli* TP has been studied, which shares 39% sequence identity with human TP [3]. The similarity between *E. coli* TP and human TP sequences is predominantly conserved in the regions containing the binding sites for thymidine and phosphate, showing 74% and 60% identity, respectively [26]. Less conserved regions (C- and N-terminal segments) are distant from the active site cleft. Therefore, *E. coli* protein can reasonably be used as a model for studies on the interaction between TP and the substrate analogues. TP and 5-fluorouracil (5-FU) were purchased from Sigma Aldrich (Germany). 5-Fluorouracil derivatives were synthesized following a procedure already published [15]. EDTA, NaCl, $NaH_2PO_4 \cdot H_2O$, $Na_2HPO_4 \cdot 7H_2O$ used for the preparation of phosphate buffer (pH 7.4) were purchased from Sigma Aldrich. Spectroscopic grade chloroform, methanol and ethanol were purchased from Carlo Erba (Milano, Italy). Milli-Q previously distilled water was used for the preparation of phosphate buffer.

2.2. Methods

2.2.1. Protein Purification

TP stock solution contains uracil 2 mM to stabilize the protein. For this reason, the solution was purified before use by filtration through the membrane of a Vivaspin Concentrator (Vivaspin 2, Sartorius Stedin, Goettingen, Germany) using a cut off of 30000 MWCO (Da), in order to keep the protein and remove only the uracil. 1 mL of phosphate buffer was added at each filtration cycle. An ALCPK 121 centrifuge (Milano, Italy) was used for 3 min at 3800 rpm for the first cycle and for 5 min at 7000 rpm for the subsequent cycles. The filtration process was repeated several times until the disappearance of the UV–Visible uracil absorption at 260 nm in the eluted fraction.

2.2.2. Liposome Preparation

The proper amount of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 25 mg/mL in chloroform purchased from Avanti Polar Lipids