Colloids and Surfaces B: Biointerfaces 140 (2016) 121–127

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Kinetics and mechanistic study of competitive inhibition of thymidine phosphorylase by 5-fluoruracil derivatives

Manuela Petaccia[a], Patrizia Gentili[b], Neva Bešker[c], Marco D'Abramo[b], Luisa Giansanti[a,*], Francesca Leonelli[d], Angela La Bella[b], Denise Gradella Villalva[b], Giovanna Mancini[e]

[a] Dipartimento di Scienze Fisiche e Chimiche, Università degli Studi dell'Aquila, Via Vetoio, 67100 Coppito (Aq), Italy
[b] Dipartimento di Chimica, Università degli Studi di Roma "La Sapienza", P.le Aldo Moro 5, 00185 Roma, Italy
[c] CINECA, SCAI—Super Computing Applications and Innovation Department, Via dei Tizii, 6, 00185 Rome, Italy
[d] Dipartimento di Biologia Ambientale, Università degli Studi di Roma "La Sapienza", P.le Aldo Moro 5, 00185 Roma, Italy
[e] CNR—Istituto di Metodologie Chimiche, Via Salaria km 29.300, Monterotondo Scalo, 00016 Rome, Italy

ARTICLE INFO

Article history:
Received 2 October 2015
Received in revised form 1 December 2015
Accepted 13 December 2015
Available online 17 December 2015

Keywords:
5-Fluorouracil
Polyoxyethylenic spacer
Liposomes
Enzymatic inhibition

ABSTRACT

In a previous investigation, cationic liposomes formulated with new 5-FU derivatives, differing for the length of the polyoxyethylenic spacer that links the N^3 position of 5-FU to an alkyl chain of 12 carbon atoms, showed a higher cytotoxicity compared to free 5-FU, the cytotoxic effect being directly related to the length of the spacer. To better understand the correlation of the spacer length with toxicity, we carried out initial rate studies to determine inhibition, equilibrium and kinetic constants (K_I, K_M, k_{cat}), and get inside inhibition activity of the 5-FU derivatives and their mechanism of action, a crucial information to design structural variations for improving the anticancer activity. The experimental investigation was supported by docking simulations based on the X-ray structure of thymidine phosphorylase (TP) from *Escherichia coli* complexed with 3′-azido-2′-fluoro-dideoxyuridin. Theoretical and experimental results showed that all the derivatives exert the same inhibition activity of 5-FU either as monomer and when embedded in lipid bilayer.

1. Introduction

5-Fluorouracil (5-FU) is a chemotherapeutic agent extensively employed in the treatment of a wide range of solid malignant tumors [1] and is the target of three specific enzymes involved in the metabolism of pyrimidines, namely thymidylate synthase, thymidine phosphorylase (TP) and dihydropyrimidine dehydrogenase. 5-FU and its derivatives exert their cytotoxic activity altering the biosynthesis of thymidine by hampering the enzymatic conversion of thymine (ribosylation and phosphorylation) to the nucleotide, thus inhibiting the normal function of DNA and RNA and, as a consequence, cell proliferation [2,3]. However, 5-FU exhibits severe side effects because of its low tumor affinity, its narrow therapeutic window and its efficacy is seriously reduced because of drug resistance [4]. Moreover, it has a short half-life (10–20 min) either in the tissue and systemically [5], its bioavailability is low (less than 20% of an injected dose undergoes enzymatic activation) [6] and unpredictable [7] because of its high variability in enzymatic degradation strictly linked to individual patient characteristics [8]. As a consequence, tumor cells are exposed to the action of the drug only for a short period. It follows that the prolonged maintenance of high serum 5-FU levels after its administration is crucial for the clinical efficacy of the therapy [9]. In recent years many efforts have been made to develop new protocols for administration and delivery of 5-FU and its derivatives, with the aim of improving their absorption and their efficacy while reducing their side effects. Hence a large number of 5-FU derivatives characterized by better features with respect to 5-FU, such as reduced toxicity, increased bioavailability, selectivity to target tissue and efficacy, to be administered alone or combined with other anticancer drugs, have been prepared [4]. We recently investigated *in vitro* the cytotoxic activity of cationic liposomes including 5-FU derivatives **1**, **2** or **3** (Chart 1) on colon cancer cells [10]. The three 5-FU derivatives differ for the length of the polyoxyethylenic spacer that links the N^3 position of 5-FU to an alkyl chain of 12 carbon atoms. It was shown that the investigated liposome formulations show a higher cytotoxicity compared to free 5-FU and that the efficacy of the formulation increases as a function of the length of the hydrophilic spacer. These results induced us to carry out further investigations to deepen our knowledge of the ability of these 5-FU derivatives to inhibit the

* Corresponding author.
E-mail address: luisa.giansanti@univaq.it (L. Giansanti).

http://dx.doi.org/10.1016/j.colsurfb.2015.12.020
0927-7765/

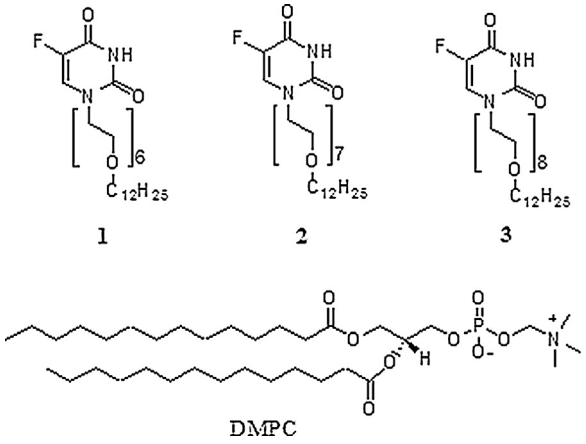

Chart 1. Molecular structure of inhibitors and DMPC.

nucleotide metabolism, and of their mechanism of inhibition of TP. Herein we report the study of the inhibitory effect of 5-FU derivatives **1**, **2** or **3** on TP activity, both used as monomers and included in liposomes composed of dimyristoyl-*sn*-glycero-phosphocholine (DMPC, Chart 1). TP was chosen for this investigation because it is the rate limiting enzyme in the intracellular enzymatic conversion of 5-FU into specific cytotoxic nucleotides or deoxynucleotides. [11,12]. Further, TP is directly involved in tumor vascularization [13,14] and favors metastasis formation [15]. As a consequence, TP inhibitors could be very useful to clarify the role of the enzyme in tumor progression and have a great potential as anticancer drug also because of their angiogenic and antimetastatic effect.

The efficacy of **1**, **2** and **3** in inhibiting TP activity and the mechanism of inhibition were investigated by initial rate kinetic experiments in different concentration conditions, and the results were compared with those obtained in the inhibition of TP activity by 5-FU. Docking simulations, based on the X-ray structure of thymidine phosphorylase (TP) from *Escherichia coli* complexed with 3′-azido-2′-fluoro-dideoxyuridin were also carried out to investigate at the molecular level the interaction of **1**–**3** derivatives with TP. A deep understanding of the mechanism of action of the investigated 5-FU derivatives, is crucial for the design and development of new efficient compounds.

2. Materials and methods

2.1. Materials

DMPC (purity >99%) was purchased from Avanti Polar Lipids (Alabaster, AL). 5-FU derivatives **1–3** were prepared and purified as previously described [10]. KH_2PO_4 was purchased from Riedel-de Haën, 5-FU, thymidine, thymine (purity >99%) and TP derived from *E. coli* (≥500 U/mL) were purchased from Sigma–Aldrich.

2.2. Liposome preparation

A lipid film was prepared on the inside wall of a round-bottom flask by evaporation of $CHCl_3$ solutions containing the proper amount of lipids (DMPC, DMPC/**1–3** at a 9/1 molar ratio). The obtained films were stored overnight under reduced pressure (0.4 mbar) and KH_2PO_4 buffer (0.1 M at pH 7.4, PB) was added to the lipid film to obtain a liposome dispersion at the desired lipid concentration. The solutions were heated at 50 °C, vortex-mixed and freeze–thawed six times from liquid nitrogen to 50 °C. Dispersions were then extruded (10 times) through a 100 nm polycarbonate membrane (Whatman Nucleopore). Extrusions were carried out at 40 °C using a 2.5 mL extruder (Lipex Biomembranes, Vancouver, Canada).

2.3. UV–vis measurements

Absorption spectra of thymidine and thymine in PB (0.1 M, pH 7.4) at concentration between $10^{-4} \div 10^{-5}$ M were recorded at 25.0 °C using a VARIAN Cary 300 UV–vis spectrophotometer, to determine the molar extinction coefficients (ε). A value of $\Delta\varepsilon = \varepsilon_{thymidine} - \varepsilon_{thymine} = 509\ M^{-1}\ cm^{-1}$ for the absorbance difference of thymidine and thymine at 290 nm was determined from absorbance *vs* thymidine and thymine concentration plots, respectively. All experiments were carried out at 290 nm to avoid interferences of 5-FU and its derivatives. The molar concentration of the TP was evaluated by UV measurements at 280 nm. The $\varepsilon \approx 24400\ M^{-1}\ cm^{-1}$ of the enzyme [16] was estimated by the summation of the ε of the absorbing amino acid (2 tryptophan residues $\varepsilon = 5500\ M^{-1}\ cm^{-1}$, 9 tyrosine residues, $\varepsilon = 1490\ M^{-1}\ cm^{-1}$) included in the amino acid sequence of the enzyme.

2.4. Determination of Michaelis constant (K_M) and k_{cat} for the phosphorolysis of thymidine

To evaluate the enzymatic activity, a modified spectrophotometric assay based on the method developed by Krenitsky and Bushby was used [17]. The enzymatic reaction was started by addition of enzyme (30 μL of a solution composed of 1.5 U of TP in 1 mL of phosphate buffer 0.10 M, pH 7.4) into a cuvette containing thymidine at different concentrations ($0.075 \div 1.0$ mM) in 3.0 mL of PB. The final molar concentration of TP was 6 nM. After mixing, the decrease in absorbance at 290 nm was monitored at 1 min intervals for 20 min at 25.0 °C. All experiments were carried out in triplicate. Initial rates of reaction were calculated from the linear range of absorbance *vs.* time plots, using a value of $\Delta\varepsilon = 509\ M^{-1}\ cm^{-1}$ (at 290 nm) for thymidine/thymine pair. Plotting initial rates *vs.* thymidine concentrations gave a Michaelis–Menten curve. Maximum rate (V_{max}), Michaelis (K_M) and catalytic constants (k_{cat}) were determined by fitting experimental points with Eq. (1):

$$V = \frac{V_{max}[S]}{(K_M + [S])} \quad (1)$$

2.5. Enzyme inhibition study

Inhibition experiments were carried out to evaluate the capability of 5-FU, **1**, **2**, **3**, DMPC/**1**, DMPC/**2** or DMPC/**3** liposomes to inhibit the activity of TP on the phosphorolysis of thymidine. The enzymatic reaction was monitored as described in the previous paragraph adding TP (30 μL of 1.5 U of TP/mL phosphate buffer solution) into cuvettes containing a fixed concentration of substrate (*i.e.*, 0.10 mM thymidine) and different concentrations ($0 \div 40$ μM) of inhibitor (in the case of **1**–**3** as monomers or included in liposome bilayers) in 3 mL of PB. An analogous experiment was performed using DMPC liposomes devoid of 5-FU derivatives. Results of the inhibition experiments were compared with uninhibited enzymatic assay performed in the same experimental conditions (see previous paragraph). To calculate inhibition effect, the activity of enzyme in the presence of inhibitor was determined by initial rate for each inhibitor concentration and then converted to percentage of inhibition of the enzyme using the Eqs. (2)–(3):

$$\text{Activity} = \frac{\text{initial rate of inhibited reaction}}{\text{initial rate of uninhibited reaction}} \times 100 \quad (2)$$

$$\text{Inhibition} = 100 - \text{Activity} \quad (3)$$