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Design and synthesis of novel 5,6-disubstituted uracil derivatives as potent inhibitors of thymidine phosphorylase

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Abstract—We report on a series of novel 5,6-disubstituted uracils with significant inhibitory activity against human and *Escherichia coli* thymidine phosphorylases. Bis-uracil conjugates were identified as the most potent inhibitors of TPs in this study.

The reversible phosphorolysis of thymidine to thymine and 2-deoxy-D-ribose-1-phosphate represents an important salvage pathway of the 2′-deoxyribonucleosides in the intracellular metabolism. This process is catalyzed by thymidine phosphorylase (TP), an enzyme, which is identical with the platelet-derived endothelial cell growth factor (PD-ECGF) that belongs to the group of proteins promoting angiogenic activity. Angiogenic factor TP stimulates endothelial cell migration from tissues due to chemotactic 2-deoxy-D-ribose, dephosphorylated product of 2-deoxy-D-ribose-1-phosphate. Degradation of thymidine by TP is also required for the inhibition of hypoxia-induced apoptosis. Moreover, thymidine phosphorylase is identical with polypeptide growth inhibitor gliostatin, which acts on astrocytes and astrocytoma cells.[1]

Angiogenesis plays an important role in the growth and metastasis of solid tumors. Therefore, inhibitors of TP have been studied extensively in the past few years. Several analogues of uracil have been proved to be potent TP inhibitors.[2,3] Nevertheless, no substance has passed the clinical trials so far; therefore, there is a pressing need to design novel analogues as potential inhibitors of TP.

Our work is focused on the synthesis of novel 5,6-disubstituted uracil derivatives. According to previous studies,[3,4] a halogen atom at the position 5 can significantly increase the inhibitory activity. On the other hand, the elucidation of optimum character of the substitution at the position 6 is non-existent. The aim of our effort was to enhance the inhibitory activity of 6-amino-5-chlorouracil (6A5CU),[5] which is known as a potent inhibitor of TP, by substitution of the amino group with different amines. We compared the inhibitory potency of these uracil derivatives toward human and *Escherichia coli* thymidine phosphorylases.

We employed a simple method for the synthesis of compounds **1**–**22** based on a selective nucleophilic substitution of the chlorine atom at the position 6 of 5,6-dichlorouracil[6] or 5-bromo-6-chlorouracil[7] by various amines (Table 1) and diamines (Table 2).[8] Reaction with diamines resulted in bis-uracil derivatives even if an excess of the starting diamine was used. Also, we introduced a novel effective method for preparation of 6-amino-5-chlorouracil hydrochloride from 5,6-dichlorouracil.[9]

All new compounds were fully characterized on the basis of their ^{1}H NMR and ^{13}C NMR spectroscopic and analytical data.[10]

The inhibitory potency of novel 5,6-disubstituted uracils on the phosphorolysis of thymidine was evaluated using two recombinant thymidine phosphorylases of a commercial origin and the enzyme from human placenta. Thymidine phosphorylase from human placenta was purified according to Yoshimura et al.[11] with minor modification (additional chromatography on Q Sepharose) and the

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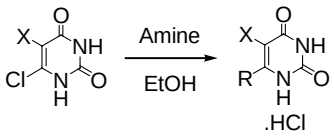
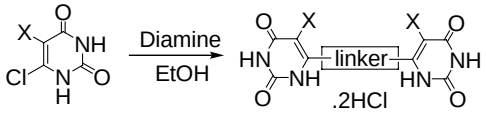
Table 1. Synthesis of uracils **1**–**20** and their inhibitory potency against thymidine phosphorylases from different sources



Compound	Amine	R	X	IC_{50}[a] (μM)		
				Escherichia coli	Human, expressed in V79 cells	Human, purified from placenta
6A5CU	NH_3	NH_2	Cl	4.0 ± 0.2	11.6 ± 0.9	8.3 ± 0.3
1	Piperidine	Piperidin-1-yl	Br	9.1 ± 0.3	8.2 ± 0.2	5.8 ± 0.2
2	Pyrrolidine	Pyrrolidin-1-yl	Br	5.3 ± 0.5	5.1 ± 0.5	4.6 ± 0.4
3	Azetidine	Azetidin-1-yl	Br	2.0 ± 0.1	6.8 ± 0.5	6.3 ± 0.5
4	Hydrazine	Hydrazino	Br	4.3 ± 0.3	3.8 ± 0.4	4.3 ± 0.3
5	Morpholine	Morpholin-4-yl	Br	11.6 ± 0.7	7.2 ± 0.6	6.5 ± 0.5
6	Cyclopropylamine	Cyclopropylamino	Br	3.5 ± 0.1	7.2 ± 0.3	7.4 ± 0.3
7	2-(Hydroxymethyl) piperidine	2-(Hydroxymethyl) piperidin-1-yl	Br	6.5 ± 0.5	7.0 ± 0.5	6.0 ± 0.5
8	3-(Hydroxymethyl) piperidine	3-(Hydroxymethyl) piperidin-1-yl	Br	6.5 ± 0.3	5.2 ± 0.4	5.0 ± 0.4
9	4-(Hydroxymethyl) piperidine	4-(Hydroxymethyl) piperidin-1-yl	Br	5.5 ± 0.5	7.5 ± 0.5	6.0 ± 0.5
10	4-(Hydroxyethyl) piperidine	4-(Hydroxyethyl) piperidin-1-yl	Br	6.1 ± 0.3	5.0 ± 0.3	4.6 ± 0.2
11	Piperidine	Piperidin-1-yl	Cl	7.3 ± 0.7	15.7 ± 0.3	12.1 ± 0.3
12	Pyrrolidine	Pyrrolidin-1-yl	Cl	4.4 ± 0.4	9.5 ± 0.3	7.4 ± 0.6
13	Azetidine	Azetidin-1-yl	Cl	14 ± 0.8	8.0 ± 0.5	7.0 ± 0.5
14	Hydrazine	Hydrazino	Cl	9.1 ± 0.6	10.2 ± 0.3	4.3 ± 0.1
15	Morpholine	Morpholin-4-yl	Cl	10.4 ± 0.6	8.3 ± 0.7	8.0 ± 0.7
16	Cyclopropylamine	Cyclopropylamino	Cl	10.1 ± 0.9	7.0 ± 0.5	6.0 ± 0.5
17	2-(Hydroxymethyl) piperidine	2-(Hydroxymethyl) piperidin-1-yl	Cl	4.3 ± 0.3	8.0 ± 0.5	8.8 ± 0.6
18	3-(Hydroxymethyl) piperidine	3-(Hydroxymethyl) piperidin-1-yl	Cl	5.4 ± 0.4	9.0 ± 0.6	7.4 ± 0.4
19	3-(Hydroxymethyl) piperidine	3-(Hydroxymethyl) piperidin-1-yl	Cl	7.5 ± 0.7	8.5 ± 0.5	6.3 ± 0.3
20	4-(Hydroxyethyl) piperidine	4-(Hydroxyethyl) piperidin-1-yl	Cl	5.5 ± 0.3	7.5 ± 0.5	9.0 ± 0.8

[a] Values represent means of minimum three experiments (standard deviations in parentheses).

Table 2. Synthesis of bis-uracils **21**–**23** and their inhibitory potency against thymidine phosphorylases from different sources



Compound	Diamine	Linker	X	IC_{50}[a] (μM)		
				Escherichia coli	Human, expressed in V79 cells	Human, purified from placenta
21	Piperazine	Piperazine-1,4-diyl	Br	2.3 ± 0.2	4.0 ± 0.3	3.6 ± 0.3
22	1,2-Diaminoethane	Ethylenediamino	Br	2.5 ± 0.2	3.4 ± 0.3	2.2 ± 0.2
23	Piperazine	Piperazine-1,4-diyl	Cl	8.7 ± 0.6	7.2 ± 0.7	3.8 ± 0.2

[a] Values represent means of minimum three experiments (standard deviations in parentheses).

hydroxyapatite fraction was used for the inhibition experiments. The standard reaction mixture (50 μL) contained 100 μM [^{3}H-methyl]thymidine, 200 μM potassium phosphate buffer, pH 6.7, and enzyme (6.7 mU TP from *E. coli*, Sigma T 2807; 0.41 mU TP human, expressed in V79 Chinese hamster cells, Sigma T 9319; 0.45 mU TP purified from human placenta[11] in 20 mM bis Tris–HCl, pH 6.4, 1 mM EDTA, and 2 mM DTT). The reaction was carried out at 37° for 10 min and stopped by spotting a 2 μL aliquot onto a Silica gel 60 F_{254} plate that had been prespotted with 0.01 μmol of each thymine and thymidine. The plate was developed in the solvent system ethyl acetate/water/formic acid (60:35:5). The spots were visualized under UV light (254 nm) and cut out for radioactivity determination in the toluene-based scintillation cocktail.

The results show that all compounds of this series efficiently inhibit at 0.1 mM thymidine and 0.2 mM potassium phosphate buffer bacterial and/or human TPs with IC_{50} values in the concentration range from 2 to 16 μM (Tables 1 and 2). At higher phosphate concentration (1 mmol/L) in the reaction mixture the IC_{50} values are about 50% higher (data not shown). Within the 5-Br series, the inhibitory effect of these 5,6-disubstituted uracils toward human TP from placenta is decreasing in the order of dependence of a substitution at the position 6: hydrazino ⩾ pyrrolidin-1-yl ⩾ azetidinyl-1-yl ⩾ piperidin-1-yl ⩾ hydroxyethylpiperidin-1-yl ⩾ hydroxymethylpiperidin-1-yl ⩾ cyclopropylamino ⩾ morpholin-1-yl ⩾ amino. In these cases, the 5-bromo derivatives are more potent compared to their 5-chloro counterparts. The inhibitory

potency of mentioned compounds toward human TP expressed in V79 cells is more or less the same with one exception: 5-chloro-6-hydrazinouracil is 2-fold less efficient inhibitor compared to the enzyme from human placenta. Different inhibitor pattern was found for *E. coli* thymidine phosphorylase where 6-azetidin-1-yl-5-bromouracil and 5-bromo-6-(cyclopropylamino)uracil possess inhibitory efficacy higher than those of 5-bromo-6-hydrazinouracil and/or 6-amino-5-chlorouracil.

5-Bromo-bis-uracil derivatives with piperazine-1,4-diyl and ethylenediamino linkers inhibit human TPs and *E. coli* enzyme with efficacy higher than those of both the paternal compound 6-amino-5-chlorouracil and 5-bromo-6-hydrazinouracil. These compounds are the most potent TP inhibitors comprised in the studied group.

In accordance with the proposed model of the enzyme–inhibitor interaction,[12] we suppose that one of the nitrogen atoms in the linker interacts with the serine oxygen of the enzyme (S117), thus, enhancing the inhibitory activity of bis-uracil derivatives. The role of the second uracil moiety is not completely clear but could enhance the probability of interaction with the enzyme.

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8. General procedure for the preparation of substituted uracils: the appropriate amine (1.0 mmol) was added to a solution of 5-bromo-6-chlorouracil or 5,6-dichlorouracil (0.45 mmol) in ethanol (4.5 mL). The resulting mixture was stirred at room temperature overnight. The precipitate was filtered off, washed with ethanol and ether, and dried in vacuo.
9. 6-Amino-5-chlorouracil hydrochloride: 5,6-dichloro uracil (0.3 g, 1.66 mmol), liquid ammonia (10 mL), and ethanol (5 mL) were placed in an autoclave and heated for 16 h at 80 °C. The excess ammonia was evaporated and the resultant precipitate was collected by filtration. The product was washed with ethanol and ether, and dried to give analytically pure white crystals (0.21 g, 64%). Mp: >300 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.10 (br s, 1H, NH), 7.00 (br s, 4H, NH); ^{13}C NMR (125.7 MHz, DMSO-d_6) δ 161.52, 157.39, 156.03, 97.21. Anal. Calcd for $C_{12}H_{14}Cl_4N_6O_4$: C, 32.17; H, 3.15; N, 18.76; Cl, 31.65. Found: C, 32.13; H, 3.05; N, 18.47; Cl, 31.49.
10. For compound **1**: Mp: 187–189 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.10 (br s, 1H, NH), 8.40 (br s, 2H, NH), 3.02 (t, J = 5.6 Hz, 4H, NCH_2), 1.65 (m, 4H, CH_2), 1.55 (m, 2H, CH_2); ^{13}C NMR (125.7 MHz, DMSO-d_6) δ 161.84, 159.02, 156.515, 87.31, 43.915, 22,39, 21.82. Anal. Calcd for $C_9H_{13}BrClN_3O_2$: C, 34.81; H, 4.22; N, 13.53. Found: C, 34.72; H, 4.07; N, 13.40.
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