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Bioorganic & Medicinal Chemistry 16 (2008) 3866–3870
Bioorganic & Medicinal Chemistry

Kinetic parameters and recognition of thymidine analogues with varying functional groups by thymidine phosphorylase

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Received 27 November 2007; revised 21 January 2008; accepted 23 January 2008
Available online 12 February 2008

Abstract—Thymidine phosphorylase (TP, EC 2.4.2.4) recognized the structure of the substrate with high specificity, via both the base and the ribosyl moieties. The replacement of 3′-OH of thymidine markedly influenced its catalytic activity with TP. The conversion of pyrimidine nucleosides with modified base moieties to the corresponding 1-phosphate form was poor. The leaving group activity decreased with an increase in aromaticity of the pyrimidine base moiety, because of increased difficulty in polarizing the base by the amino acids local to the active site. The replacement of 3′ and 5′ functional groups tended to decrease the reaction rate and the percentage conversion with TP. In particular the ribosyl 3′ hydroxyl group was structurally important for the binding of the substrate by the enzyme. The kinetic assay clearly showed high K_m and low V_{max} values on replacing the 3′ hydroxyl group with hydrogen.

1. Introduction

Thymidine phosphorylase (TP, EC 2.4.2.4) is a unique metabolic control enzyme, which converts thymidine to 2-deoxy-D-ribose-1α-phosphate and thymine base in phosphate buffer (Scheme 1). TP can effect the transglycosylation of pyrimidine nucleosides, and thus it is available to synthesize unnatural nucleosides.[1,2] Additionally, it is known that TP is associated with tumor-dependent angiogenesis.[3–5] Inhibition of this enzyme could potentially be useful in treating tumors depending on the nucleoside salvage pathway for their proliferation and vascularization[6–10].

Thymidine phosphorylase is a dimeric enzyme made up of two identical subunits.[11] Human TP shares 39% of its sequence identity with *Escherichia coli* TP giving rise to significant sequence homology.[12] The mechanism of the phosphorolysis reaction is as follows: the ribosyl 1-anomeric carbon undergoes nucleophilic attack at its α face by a phosphate ion.[13,14] Norman et al. reported the crystal structure of TP complexed with TPI (5-chloro-6-[1-(2-iminopyrrolidinyl)methyl]uracil) HCl in an active conformation.[15] This inhibitor has been proposed as a transition-state analogue of TP, assuming that the transition-state structure is oxonium-like and that the protonated pyrrolidine is an oxonium mimic. However, this structure of TP complexed with an inhibitor offers no direct insights as to the basis of specific interactions between TP and the ribosyl moiety.

Here, we report the reactivity and kinetics of thymidine phosphorylase (from *E. coli*) using thymidine analogues. Our research proves the influence of the functional groups and the ribosyl structure on the ability of TP to discriminate between substrates, and it will provide the basis for further drug design studies with the aim of increasing inhibitor potency and specificity.

2. Results and discussion

Table 1 shows the effect of varying the base moiety of the substrate in the TP-catalyzed reaction. The substrates possessing thymine base **1** or uracil base **4** reacted easily, and the reactivity of each substrate was approximately the same. The thymidine of α-anomer base **5** did not react at all. This enzyme recognized the configuration of the 1′-anomer position for the pyrimidine nucleoside

Keywords: Thymidine phosphorylase; Kinetic parameters; Substrate recognition; Thymidine analogues.
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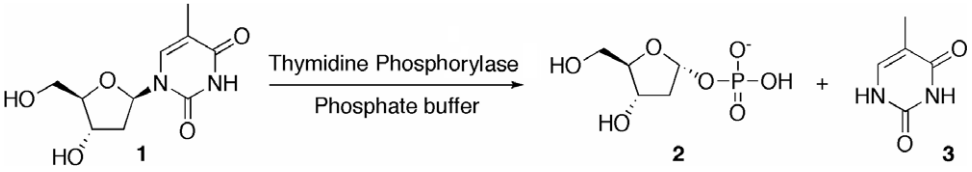
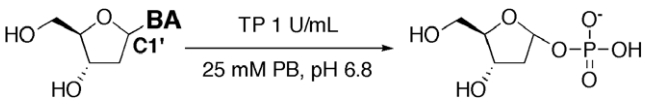
doi:10.1016/j.bmc.2008.01.038



Scheme 1. Phosphorolysis reaction of thymidine with thymidine phosphorylase.

Table 1. Conversions of thymidine analogues with varying the base moiety with TP



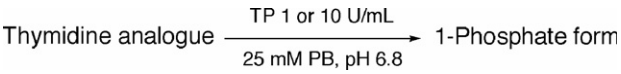
Base analogue	C_1'	Conversion (%)	
		2 h	24 h
1	β	86	82
4	β	88	83
5	α	0[a]	0[b]
6	β	0[a]	0[b]
7	β	0[a]	0[b]
8	β	5.4[a]	7.9[b]

[a] 10 U/mL, 24 h.
[b] 10 U/mL, 1 week.

with high specificity. 5-Methyl-2-pyrimidinone nucleoside **6**[16,17] and deoxycytidine **7** were unreactive though the substrates have β-anomer structures. These pyrimidine compounds showed lower leaving activities than thymine base. As the aromaticity of the pyrimidine moiety increases, the leaving activity decreases, because of increasing difficulty in polarizing the base by the amino acids of the active site. 6-Azathymidine **8** was converted to deoxyribose-1-phosphate in 5.4% yield during 24 h (TP concentration was 10 U/mL). The replacement of C-6 of this base with a nitrogen atom greatly decreased its reactivity. The recognition of the base moiety was very specific for thymidine phosphorylase. However, TP has broad specificity in relation to the recognition of the 5 position of the pyrimidine base.[2] We clarified that each of the 5-substituted uracil compounds, halogen, ethyl, amino, trifluoromethyl, reacted with thymidine to give the corresponding unnatural nucleoside in good yield.

We investigated the effect of the structure of the ribosyl ring on the TP catalyzed reaction (Table 2). Compounds **9** (β form) and **10** (α form) are 4′-thionucleosides, in which oxygen is replaced with sulfur at the ribosyl 4′ position of thymidine **1**.[18,19] The structure of the ribosyl ring of 4′-thiothymidine **9** is different from native substrate **1**, because

Table 2. Effect of the ribosyl skeleton of the substrate on reaction with TP



Thymidine analogue	Conversion (%)
1	86[a]
9	3.8[b]
10	0[b]
11	0[b]
12	0[b]

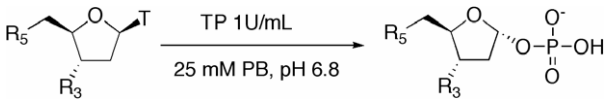
[a] Reaction time: 1 h, TP; 1 U/mL.
[b] Reaction times: 24 h, TP; 10 U/mL.

the atomic radius of sulfur is larger than that of oxygen. The reactivity of compound **9** was very low, and the conversion was 3.8% with 10 U/mL of thymidine phosphorylase during 24 h. Our experiment proved that TP specifically recognizes the size of the ribosyl skeleton of the substrate. 4′-Thio-1′α-thymidine **10** could not be converted to the 1-phosphate form and a free thymine base. The base of the α form did not fit in the pocket of TP as was observed in the reaction of **5**.

1-{[2-Hydroxy-1-(hydroxymethyl)ethoxy]methyl}thymine **11** is a thymidine analogue possessing a flexible backbone.[20,21] This compound bears a structure which is analogous to an open ribosyl moiety, and could not construct the oxonium intermediate by means of complexing the enzyme. TP could not convert compound **11** into thymine base **3** and the corresponding phosphate form. In compound **12** the ribosyl skeleton was replaced with a hexopyranosyl ring.[22] It was found that although compound **12** was capable of forming an oxonium intermediate, complexation with TP failed. This was most likely due to the backbone of **12** being larger than that of native substrate **1**, giving rise to conformational differences between this analogue and the active site of TP. Therefore, it can be concluded that the ribosyl ring is necessary in the recognition of the substrate structure.

Table 3 shows the effect of different functional groups on the ribosyl moiety on the TP catalytic reaction. The hydroxyl groups at 3′ and 5′ positions were variously replaced by hydrogen, amino, thiol, halide or azide groups. The replacement of the 3′ and 5′ groups decreased the reaction rate and the percentage conversion with TP. The replacement of 3′-OH group had a profound effect on the reactivity; reaction of 3′-deoxythymidine **15**[23] proceeded with an initial rate of 0.74 μM/min and went to 3.2% conversion. These values were lower than those of 5′-deoxythymidine **14**.[24] Compound **14** proceeded with an initial rate of 120 μM/min and went to 68% conversion after 1 h. In addition to these, 3′β-thymidine **13**, in which the configuration of the 3′ hydroxyl group is inverted relative to **1**, failed to react under the same conditions. As also demonstrated by the replacement with the other functional groups –NH_2, –SH groups (**17**,[25,26] **19**[27]), the 3′ replacement appeared to significantly influence the reaction with TP. These results prove that the 3′ hydroxyl group interacts with the amino

Table 3. Conversions and initial velocities of thymidine analogues with varying functional groups on the ribosyl moiety with TP



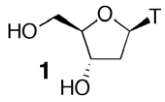
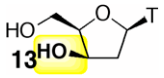
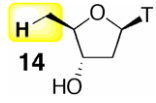
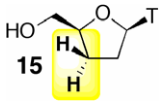
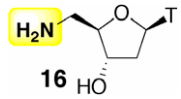
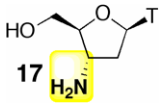
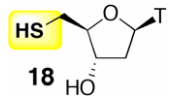
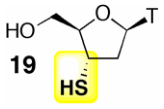
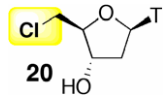
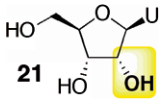
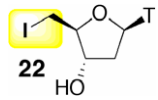
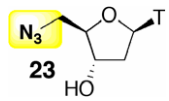
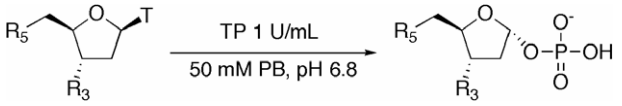
Substrate	Conversion (%)		v_0 (μM min^{-1})	Substrate	Conversion (%)		v_0 (μM min^{-1})
	1 h	24 h			1 h	24 h	
 **1**	86	91	1000	 **13**	0	0	—
 **14**	68	91	120	 **15**	3.2	6.1	0.74
 **16**	11	36	4.9	 **17**	0.76	1.5	0.013
 **18**	39	90	11	 **19**	0	0	—
 **20**	24	70	5.5	 **21**	56	81	12.3
 **22**	0	0	—				
 **23**	0	0	—				

Table 4. Kinetic parameters for thymidine analogues varying the ribosyl hydroxyl groups with thymidine phosphorylase



Thymidine analogue	R_3	R_5	K_m (mM)	V_{max} (mM/min)
1	–OH	–OH	2.5	5.2
14	–OH	–H	10	3.4
15	–H	–OH	32	0.014

acid residues of TP, and selectively binds the substrate. The analogues (**16**,[26] **18**,[28] **20**[24]) in which the 5′ group is substituted had less influence on the final conversion, however, the initial rates decreased in comparison with that of the native substrate **1**. Compounds **22**[24] and **23**,[26] with their 5′ positions modified with an iodide or azide group, respectively, were insoluble in phosphate buffer (pH 6.8) at 35 °C, and these substrates were not converted to the 1-phosphate form with TP. A 2′ hydroxyl group (uridine **21)** resulted in higher conversion and lower initial velocity in comparison with thymidine **1**. Therefore, it can be concluded that the 3′α-hydroxyl group is very important for the recognition of the ribosyl substrate with thymidine phosphorylase.

We performed the kinetic assay of the reaction of thymidine analogues catalyzed by TP. Phosphate ion concentration was kept constant at 50 mM of phosphate buffer (pH 6.8), and we examined the effect of the position of the hydroxyl group on the ribosyl moiety on the TP catalyzed reaction. The substrates were thymidine **1** and the 3′- and 5′-deoxythymidine (**14** and **15**). Table 4 clearly indicates that thymidine **1** was the most effective among these substrates, K_m was 2.5 mM, and V_{max} was 5.2 mM/min. K_m and V_{max} for 5′-deoxythymidine **14** were 4 times higher and 0.65 times lower than those of native substrate **1**, respectively, indicating its unsuitability as a substrate. In contrast, use of 3′-deoxythymidine **15** in which 3′-OH was replaced by hydrogen H, had a profound effect on the values of K_m and V_{max}. V_{max} decreased 370 times on replacing the 3′ hydroxyl group of thymidine **1** while the K_m value of **15** increased 13-fold in comparison to substrate **1**. Thus, it can be concluded that the 3′α-hydroxyl group is very important for the recognition of the ribosyl substrate. The substitution of the ribosyl 5′-OH only slightly retarded the catalytic reaction with TP.

3. Conclusion

Our results led us to conclude that not only the base moiety but also the ribosyl moiety was important for the substrate recognition of thymidine with thymidine phosphorylase. It is acceptable to vary the group at the 5 position of the thymine base, however, substitution at the 6 position of the pyrimidine has a profound effect on the leaving group. The 6-azathymidine, in which C-6 was replaced by a nitrogen atom, converted very slowly to the corresponding nucleoside. It was found that the leaving ability of the base moiety was lowered by an increase in aromaticity of the pyrimidine ring. Phosphorylation of ribosyl 1′ position was drastically effected in the absence of the 3′α-hydroxyl group, because it was necessary for the enzyme to be able to bind the substrate for nucleophilic attack. It is obvious that the 5′-OH group does not interact with the active site of the enzyme. The ribosyl skeleton also effected the reactivity with TP. 4′β-Thiothymidine **9** reacted slowly with TP, because the ribosyl ring was much wider than that of the native substrate. The hexopyranosyl compound **12** does not fit in the active site of the enzyme, as the pyranosyl skeleton is larger than that of thymidine. The ribosyl skeleton was shown to be very important for the recognition of the substrate by thymidine phosphorylase. The kinetic assay clearly showed that a 3′-OH group on the ribosyl moiety was indispensable for binding the substrate of TP.

4. Experimental

4.1. General

The synthesis and characterization of thymidine analogues used in the experiments have been previously reported: **6**,[16,17] **9** and **10**,[18,19] **11**,[20,21] **12**,[22] **14**,[24] **15**,[23] **16**,[26] **17**,[25,26] **18**,[28] **19**,[27] **20**,[24] **22**,[24] **23**.[26] All solvents and reagents were of reagent-grade quality, and used without further purification. All thymidine analogues were characterized using ^{1}H NMR, ^{13}C NMR, EIMS. Thymidine phosphorylase (from *E. coli*, EC 2.4.2.4) was purchased from Sigma–Aldrich Chemical Co.

4.2. HPLC

HPLC system was a GULLIVER model by JASCO. Each conversion was assayed with a C-18 column (250 × 4.6 mm, Shiseido AQ or GL Science Inertsil ODS-3) HPLC at a flow rate of 1.0 mL min^{-1}. The mobile phase was 0.5–10% acetonitrile in 100 mM phosphate buffer (pH 6.8). The UV detector was set at 260 nm and the column was operated at 40 °C.

4.3. Phosphorylation of thymidine analogues

Incubations were carried out on a small scale and generally contained 1 mM of the thymidine analogue, 1 U/mL of thymidine phosphorylase in 1 mL of 25 mM phosphate buffer (pH 6.8). Mixtures were stirred at 35 °C for 1 or 24 h. The detection was by UV absorption at 260 nm of thymine base produced by thymidine.

4.4. Kinetic assays

Kinetic assay was carried out on the basis of the Michaelis–Menten method, $v_0 = V_{max}[S]/(K_m + [S])$. Initial rate v_0 of each reaction was evaluated against 1 U/mL of TP over a range of concentrations with a variety of thymidine analogues at 35 °C. The phosphate ion concentration was kept constant at 50 mM. The data

from the kinetic experiments were fitted to a Lineweaver–Burk plot of 1/S versus $1/v_0$.

Acknowledgment

This research was supported partly by the Astellas Foundation for Research on Medicinal Resources and Hamamatsu Science and Technology Foundation.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.01.038.

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